Production, Purification, Characterization and Active-site Mapping of Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F

A Thesis Submitted
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Arun Goyal

to the

DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY KANPUR

May 1995

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STATEMENT

I hereby declare that the matter incorporated in this thesis entitled "**Production, purification, characterization and active site mapping of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F**" is the result of investigations carried out by me under the supervision of Professor S.S. Katiyar in the Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur, India.

In keeping with the general practice of reporting scientific observations, due acknowledgment has been made wherever the work described is based on the findings of other investigators.

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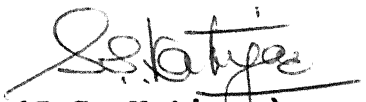

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CHM 681	Basic Biological Chemistry
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Arun Goyal
15th of May 1995

SYNOPSIS

The role of microorganisms in the biochemical transformations is known since ages and they have been used by humans since then in the preparation of food, drink and textile. With the advent of modern biotechnological techniques, it is now possible to exploit these microbial transformations in making food and dairy products, dextran, antibiotics, biofertilizers and few organic solvents. Besides these, the production of microbial enzymes and their application in the food and pharmaceutical industries have now become an important area of industrial microbiology. For example, commercial production of clinical dextran by dextransucrase released from *Leuconostoc mesenteroides*.

The extracellular dextransucrases are biosynthesized by microorganisms belonging to the family *Lactobacillaceae* and *Streptococcaceae*, especially by the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus*. The bacterium *Leuconostoc mesenteroides* belonging to family *Lactobacillaceae* is the most commonly used microorganism for production of dextransucrase and dextran. Commercial dextran is produced almost exclusively

from the bacterial strain *Leuconostoc mesenteroides* NRRL B-512F. This particular strain secretes only a single dextransucrase that produces highly uniform dextran. Dextransucrase is a member of a general class of glucosyltransferases and has been named in the literature as α,(1→6) D-glucan: D-fructose 2-glucosyltransferase [EC 2.4.1.5].

The enzyme catalyzes the synthesis of glucose polymer, dextran from sucrose following the equation;

$$n\ C_{12}H_{22}O_{11} \longrightarrow (C_6H_{10}O_5)_n + n\ C_6H_{12}O_6$$

(SUCROSE) (DEXTRAN) (FRUCTOSE)

The energy for condensation of glucosyl units is derived from the hydrolysis of sucrose. Sucrose is the only known inducer for the synthesis of dextransucrase. The dextran obtained by this strain is a homopolysaccharide containing 95% α,(1→6) glucosidic linkages in the main linear chain and remaining 5% of α,(1→4), α,(1→3) and very few α,(1→2) branched linkages. Presence of 95% linear linkages makes this dextran water soluble.

The present investigations are carried out on the production, purification, characterization and active site mapping of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. The thesis comprises of five chapters. Chapter I is the

General Introduction in which brief review of relevant literature is given pertaining to authorities of the organism, its maintenance, dextran and its uses, dextransucrase; its production, purification and properties and the mechanism of dextran synthesis. It also contains brief review of methods used for determination of total amino acid composition and elucidation of amino acid residues present at the active site of an enzyme.

The second chapter is devoted to the studies on maintenance of culture, optimization of conditions and the effect of nutrients on dextransucrase production. A variety of maintenance media were screened to select a medium for culture maintenance and capable of giving higher dextransucrase production. Modified *Lactobacillus* MRS medium proved to be the most suitable for maintenance and propagation of *Leuconostoc mesenteroides* NRRL B-512F, as it gave higher enzyme yield as compared to the other media. The temperatures in the range 20 to 30°C have been used for dextransucrase production. There are conflicting reports on the requirement of aeration and agitation of culture media for the production of dextransucrase. This led us to re-investigate and establish the extent of temperature effect on the dextransucrase production within a close range and compare the enzyme production in still and shaken flask cultures. The optimum temperature of 23°C and

still flask culture were found to be the most suitable conditions for dextransucrase production. The present study has also shown that the enzyme exhibits maximum activity at 30°C, pH of 5.2 and 10% of sucrose concentration. Various media compositions have been employed for production and improvising the yield of dextransucrase. The effect of certain nutrients on enzyme production were studied. An increase in concentration of sucrose in the enzyme production medium up to 4%, enhanced the enzyme yield. Peptone and beef extract in addition to yeast extract also improved the enzyme production. Interestingly, low concentrations of yeast extract and higher levels of phosphate favored the enzyme yield. Besides these, agents such as tween 80 and nutrients such as $MgCl_2$ and NaF also marginally enhanced the enzyme yield.

In the third chapter, purification of dextransucrase by fractionation and phase-partition methods using nonionic hydrophilic polymer polyethylene glycol (PEG) of different molecular weights have been described. This chapter also contains details of the effect of certain stabilizers on dextransucrase and the total amino acid composition of the enzyme. A simple and effective method was devised for the purification of dextransucrase by fractionation with PEG 400. It was found that fractionation in three consecutive steps with a final concentration of 33% PEG 400 reproducibly yielded a

homogeneous preparation of dextransucrase which showed a single band on analysis by SDS-polyacrylamide gel electrophoresis. The purification method gave enzyme protein having a specific activity of 29 U/mg protein and an overall yield over 70%. Dextransucrase was also purified by phase-partition method using PEG of different molecular weights. Phase-partition occurs between dextran and polyethylene glycol (PEG). The addition of PEG solution to a dextran rich aqueous solution, led to the appearance of two phases; the top phase being rich in PEG while the bottom one was rich in dextran. In three successive steps of phase-partition by PEG 6000, enzyme with specific activity of 38.7 U/mg was obtained after the third step, and the overall yield was around 78%. Although, the specific activity and yield of dextransucrase obtained by phase-partition method were higher, the purification by fractionation method was comparatively simpler, less time consuming and easy to carry out. The effects of some stabilizers and temperature conditions for storage of dextransucrase were studied. Dextransucrase was stabilized against activity losses by glycerol, PEG 8,000, dextran and tween 80. Glycerol proved to be best stabilizer of the enzyme. The amino acid composition of purified dextransucrase showed that there are only 1.5 ± 0.3 cysteine residues per enzyme molecule. This illustrated that its tertiary structure is

solely dependent on other types of secondary interactions such as hydrogen bonding, ionic and hydrophobic interactions.

The role of enzymes as biocatalysts for reactions occurring in all organisms as well as their increasing use in biotechnology has stimulated the investigations on their structural organization and mechanism of action. By the combination of reaction kinetics and chemical modification technique, a great deal of information regarding structural and functional aspects of the enzyme can be obtained. The most widely applied approach for identification of the catalytic residues is the use of chemical modification methods. This is accomplished by using reagents directed to modify distinct chemical groups in the enzyme. The goal is to produce a change in the catalytic property of the enzyme that can be correlated with the functional role of specific amino acid residues. Although work has been carried out on the mechanism of dextran synthesis and structural organization of catalytic site of dextransucrase, not much information is available on the nature of amino acids at the active site. This aspect has been studied in detail in the present work reported in chapter IV.

Fourth chapter reports the presence of an essential lysine residue at the active site of dextransucrase by chemical modification studies using lysine-specific reagents *viz.* pyridoxal 5′-phosphate (PLP) and 2,4,6-trinitrobenzenesulphonic

acid (TNBS). Dextransucrase was inactivated by both of these inhibitors. The inactivation by PLP and TNBS followed pseudo-first order kinetics. The reversible inhibition of enzyme by PLP showed that the inactivation is due to specific modification of ε-amino group of lysine and not due to non-specific interactions of the inhibitor. The presence of dextran gave very little protection to the enzyme against PLP inactivation. Protection experiments showed that the modified lysine was present at the active site. The analysis of kinetic data of inactivation using Tsou's statistical method suggested the presence of one lysine residue that is essential for the catalytic activity of enzyme. Similarly, the stoichiometry of inactivation of dextransucrase by TNBS also showed the modification of one mol of lysine per mol of enzyme. All these results established that there is one essential lysine residue at the active site of dextransucrase that is involved in catalysis.

Chapter V describes the studies on the reaction of dextransucrase with bifuncational reagent *o*-phthalaldehyde. Dextransucrase was rapidly inactivated by *o*-phthalaldehyde and the inactivation was concentration and time dependent. The reaction followed pseudo-first order kinetics. The stoichiometry of inactivation by *o*-phthalaldehyde showed, formation of 1 isoindole derivative per enzyme molecule. The

substrate sucrose, protected the enzyme against *o*-phthalaldehyde inactivation indicating that the lysine and cysteine residues are present at the active site. The reaction of dextransucrase with *o*-phthalaldehyde in the presence of β-mercaptoethanol led to the modification of 14 lysine residues. However, the rate of enzyme inactivation was similar in the absence or presence of β-mercaptoethanol. This clearly showed that lysine residues modified in the presence of β-mercaptoethanol do not contribute to the enzyme inactivation. Thus only one specific lysine residue which is proximal to cysteine is essential for catalytic activity of enzyme. Dextransucrase was not inactivated by thiol specific inhibitor, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB). This confirmed that the cysteine involved in *o*-phthalaldehyde reaction leading to isoindole formation is not required for the activity. Further, the results of inactivation and fluorescence experiments with dextransucrase pre-treated with DTNB followed by *o*-phthalaldehyde treatment clearly established that the cysteine is present in close proximity of the lysine but is not participating in the catalysis. The results of fluorescence studies on dextransucrase pre-treated with PLP followed by *o*-phthalaldehyde incubation showed that both these inhibitors are binding to the same specific lysine residue that is essential for catalytic activity of the enzyme. All the results

convincingly led to the conclusion that there is only one lysine residue which is essential for the activity of dextransucrase.

It is well established that two identical sucrose binding sites and one acceptor binding site constitute the active site of dextransucrase. Two sucrose sites are required for the synthesis of dextran whereas, only one sucrose site for the synthesis of acceptor products. The exact role of lysine in catalysis is not known but presumably the critical lysine is present between the two identical sucrose binding sites and is essential for maintaining the conformation of active site. The modification by inhibitors change the conformation of the active site so that the spatial arrangement of two sucrose binding sites are altered, leading to the enzyme inactivation.

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CHAPTER I

GENERAL INTRODUCTION

The role of microorganisms in the biochemical transformations is known since prehistoric times. Microbial processes have been used by humans since then in the preparation of food, drink and textiles. In many cases, these processes became controlled and perfected to an astonishing degree by purely empirical methods. The outstanding examples of traditional microbial processes are those used in the production of beer and wine, the pickling of certain materials, the leavening of bread, the making of vinegar, cheese and butter and the retting of flax. The rise of microbiology, which revealed the nature of these traditional processes led not only to great improvements, but also to the development of entirely new industries based on the use of microorganisms that had previously not been exploited by humans. With the advent of modern biotechnological techniques, it is now possible to exploit these microbial transformations in making alcoholic beverages, food and dairy

products, dextran, organic solvents, antibiotics and bio-fertilizers. Besides these, the production of microbial enzymes and their application in food and pharmaceutical industries have now become an important area of industrial microbiology. For example, commercial production of clinical dextran using dextransucrase released from *Leuconostoc mesenteroides*.

The role of enzymes as biocatalysts for reactions occurring in an organism as well as their increasing use in biotechnology has stimulated the investigations for the understanding of their structural organization and mechanism of action. The characteristic feature of enzymes is their specificity and ability to catalyze a reaction at a very high rate under mild conditions. Several approaches have been used to understand the enzyme mechanism. By studying the reaction kinetics one can learn about the order in which substrates bind and products are released from the enzyme. However, this does not provide information regarding the molecular details of mechanism by which these transformations take place and how the structural elements contribute to the process. By the combination of reaction kinetics with chemical modification technique a great deal of information regarding the structural and functional aspects of the enzyme can be obtained.

I.1 DEXTRAN

Dextrans are a class of polysaccharides synthesized from sucrose by bacterial enzymes (dextransucrase glucansucrases or glucosyltransferases). In 1861, Pasteur [1] had shown that thickening of cane and beet sugar juices were caused by microbial action. The term dextran was first used by Scheibler in 1874 [2], when he found that, the mysterious thickening of cane and beet sugar juices was caused by a carbohydrate of empirical formula $(C_6H_{10}O_5)$. Van Tieghem [3] named the causative bacteria *Leuconostoc mesenteroides*. Dextran is the collective name now given to a class of polysaccharides composed exclusively of the monomeric unit α-D-glucose linked mainly by α(1⟶6) bonds and a variable amount of α(1⟶2), α(1⟶3) or α(1⟶4) bonds.

The two principal genera of bacteria which produce the enzyme that synthesizes dextran are *Leuconostoc* and *Streptococcus*. The most of the recent work with dextrans has been conducted with *Leuconostoc mesenteroides* and in particular the NRRL strain B-512F as the dextran obtained by this strain is a homopolysaccharide containing 95% α,(1⟶6) glucosidic linkages in the main linear chain and remaining 5% of α,(1⟶4), α,(1⟶3) and very few α,(1⟶2) branched linkages. Presence of 95% linear linkages makes this dextran water soluble [4], which has various uses. Dextrans synthesized by dextransucrase from

other species of *Lactobacillaceae*, contain higher percentage of branched $\alpha,(1\rightarrow3)$ glucosidic linkages in the main chain and are thus, water insoluble. Around 1970, dextran was recognized to be the principal component of dental caries [4,5]. Insoluble dextran synthesized by *Streptococcus mutans* causes dental plaque that adheres to the enamel of the teeth. Dextrans from various strains of bacteria have been classified and characterized [6]. A multitude of dextrans have been obtained from a number of microorganisms [7-9] and reviewed [10-12]. The synthesis of dextran from sucrose by cell free extracts of *Leuconostoc mesenteroides* culture was first accomplished by Hehre in 1941 [13].

Series of reports are available on the production of dextran by fermentation using *Leuconostoc mesenteroides* [14-34]. Extensive work has been done on optimization and modification of the fermentation processes for improved production of dextran. Conventional fermentation used for production of the dextran involves three processes; cell growth, enzyme producing phase and dextran synthesis [28]. Sucrose solution fortified with required nutrients, are inoculated with the bacterial culture with little or no process control. This approach, which combines all those phases, has the disadvantage that culture conditions will be transitory optimal for any of these processes [28]. Since, the dextran

synthesis takes place outside the cell in presence of dextransucrase, decoupling of the enzyme and dextran production is now being explored for optimizing the dextran synthesis [23,24]. Fed batch reactor maintained at constant pH of 6.7 have been used for enhancing the production of dextran [26,30]. Other approaches include, immobilization of *Leuconostoc mesenteroides* NRRL B-512F cells on stainless steel [35], by encapsulation in calcium alginate beads [36-38] and other supports [39-42] and its subsequent use for the dextransucrase and dextran production.

A novel approach using chromatographic bioreactor-separator has been reported for the synthesis of clinical dextran by purified dextransucrase [38]. Fructose, which is the by product of dextran synthesis was retained by complexing with calcium ions on the resin used in the reactor, which facilitated the higher production of dextran having molecular weight in the range of 10,000 and 200,000 [38].

I.1.1 Uses of dextran

The importance of soluble dextran synthesized by *Leuconostoc mesenteroides* lies in its wide applications in pharmaceutical, food, agricultural and fine chemical industries. Dextran and its derivatives are used in medicine as blood plasma extenders and blood flow improver, anti-ulcer

agent, against iron deficiency anemia and in open wound healing [43]. Its derivative dextran sulfate has anti-coagulant properties although less effective than that of heparin. It also inhibits viral infections. It binds to the attenuated polio virus and interferes with its initial adsorption to susceptible cells. Sodium salt of dextransulfate is found to be the inhibitor of AIDS virus [44]. Dextran is used as preservative in food industry [43]. The derivatives of dextran like sephadex and DEAE-dextran serve as molecular sieves and are extensively used in the separation of biomolecules [43]. The dextran has agricultural applications too. The seeds can be coated with dextran film in which fungicide or any insecticide can be incorporated [43]. Dextran also finds a significant use in the photographic industry [43]. Improved effects are achieved by incorporating dextrans into X-ray and other photographic emulsions resulting in economy of silver usage without the loss of fineness of the grain.

I.2 THE MICROORGANISM

The extracellular dextransucrases are synthesized by microorganisms belonging to the family *Lactobacillaceae* and *Streptococcaceae*, especially by the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus*. Species of these genera reported to elaborate dextransucrase are listed in Table I.1.

Table I.1

Lactobacillus	*Leuconostoc*	*Streptococcus*
L. acidophilus	*L. mesenteroides*	*S. bovis*
L. brevis	*L. dextranicum*	*S. Faecalis*
L. casei	*L. citrovorum*	*S. mutans*
L. musicus		*S. salivarius*
L. pastorians		*S. sanguis*
L. viridescens		*S. sobrinus*

All the species producing dextransucrase, have one common characteristic in that sucrose is in general, the only suitable carbohydrate source for dextransucrase and dextran biosynthesis. The bacterium *Leuconostoc mesenteroides* belonging to the family *Lactobacillaceae* is the most commonly used microorganism for the production of dextransucrase and the dextran. This bacterium has been isolated from sucrose-rich food preparations, materials related to sucrose production, like juices from pressed cane, soil from the cane fields and soil in and around refineries and distillaries. It is a gram positive, non-motile, non-spore forming bacterium with spherical or lenticular cells in pair or chain. It was found that *Leuconostoc mesenteroides* of the particular strain NRRL B-512F produced enzyme in maximum yield and activity, which converts sucrose to fructose and highly uniform dextran [17].

In addition, this strain secretes only a single dextransucrase and a low amount of levansucrase [17]. Thus, commercial dextran is produced almost exclusively from the bacterial strain *Leuconostoc mesenteroides* NRRL B-512F.

I.2.1 Maintenance of the culture

Various media for the maintenance of *Leuconostoc mesenteroides* have been reported [13-17,20-22,38,45,46]. The organism can be stored and maintained in sucrose rich media [14-16,46], which are also used for isolation of *Leuconostoc mesenteroides*. Jeanes [20,21] described two different media for the maintenance of stock cultures. *Lactobacillus* MRS medium is traditionally used for maintaining all the organisms belonging to family *Lactobacillaceae* [45]. This medium is also used for long term preservation of *Leuconostoc mesenteroides*. El-Sayed *et al.* [34] described a Tomato-tryptone medium for culture maintenance. For short periods, the culture can be stored in enzyme production medium as described by Tsuchiya *et al.* [47]. Although, there are many reports suggesting different media for maintenance of the *Leuconostoc mesenteroides* NRRL B-512F no report is available on the influence of maintenance medium on enzyme production.

I.3 DEXTRANSUCRASE

Dextransucrase is a member of a general class of glucosyltransferases and has been named in the literature as $\alpha,(1\rightarrow6)$ D-glucan: D-fructose 2-glucosyltransferase [EC 2.4.1.5]. This enzyme catalyzes the synthesis of glucose polymer, dextran from sucrose according to the equation.

$$\underset{\text{(SUCROSE)}}{n\ C_{12}H_{22}O_{11}} \longrightarrow \underset{\text{(DEXTRAN)}}{(C_6H_{10}O_5)_n} + \underset{\text{(FRUCTOSE)}}{n\ C_6H_{12}O_6}$$

The energy required for the condensation of glucose residues is derived from the hydrolysis of sucrose. Sucrose is the only known inducer for the synthesis of dextransucrase [48]. The enzyme is synthesized by microorganism during the active growth phase. The enzyme has gained commercial importance because of its applications in pharmaceutical, food, agricultural and fine chemical industries.

I.3.1 Production of dextransucrase

The production of enzyme dextransucrase in sucrose broth cultures of *Leuconostoc mesenteroides* NRRL B-512F and its role in dextran synthesis was thoroughly demonstrated [13,21,29,47]. A large number of reports are available on the production of dextransucrase under different culture conditions using

different production media and using batch, fed-batch, semi-continuous and continuous processes [22-26,28-30,33,35-38, 39-42,47,49-54]. These reports show that the dextransucrase production varies under different culture conditions. It has been generally observed that higher yields of dextransucrase are obtained with fed-batch fermentation techniques [26,30,51]. The most important parameters for dextransucrase production are pH [25,28,47,52,53] and temperature of fermentation medium [28,47]. Maximal enzyme production has been observed when the fermentation was carried out at constant pH of 6.7 and temperature of 23°C [28,47]. Among other nutrients, nitrogen source used in the culture media for the growth of *Leuconostoc mesenteroides* NRRL B-512F is corn steep liquor or yeast extract. Yeast extract also provides other essential trace elements. Enzyme yields are also affected significantly by the type of yeast extract used [51]. Extensive work has been carried out on the production of dextransucrase for improvising the enzyme yield. Not much literature is available on the study of effect of other micro and macro nutrients on the production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F.

I.3.2 Purification and characterization of dextransucrase

A series of techniques have been applied for the purification of dextransucrase [40-42,50,55-59]. Different combination and sequence of techniques like ultrafiltration, salt and PEG precipitation, chromatography, phase-partition method have been applied for the purification of the enzyme. Robyt and Walseth [49] purified the enzyme by chromatography on Bio-Gel A-5m. They reported the enzyme specific activity of 53 U/mg with 33% overall yield. Kobayashi and Matsuda [55] applied a number of purification methods in sequence like hydroxy-apatite, sephadex G-100, CM-cellulose, sepharose-6B and aminobutyl sepharose-4B and obtained a specific activity of 72 U/mg with an yield of 1.9%. Kaboli and Reilly [40] used ultrafiltration and gel permeation chromatographic methods using Ultrogel AcA 34 and reported specific activity of 58 U/mg with 30% yield. Monsan and Lopez [41] reported even higher specific activity 122 U/mg with 13% yield using gel permeation chromatographic method using Ultrogel AcA-34. Monsan *et al.* [42] used ultrafiltration and gel permeation chromatography using Ultrogel AcA 34 and reported 103 U/mg specific activity with 51% total yield. However, the best results were obtained by phase-partitioning method developed by Monsan *et.al.* [42] and Paul *et.al.* [50]. They reported a high specific activity of 171 U/mg. Miller *et al.* [56] also obtained a specific activity

of 170 U/mg with 54% total yield using chromatographic techniques. Fu and Robyt [57] purified the dextransucrase by chromatography using Bio-Gel A-5m and obtained a specific activity of 84 U/mg with 42% total yield.

The enzyme remains in an aggregated form in the presence of dextran resulting in high molecular weight and exists in single or multiple forms having molecular weights in the range 64,000 - 245,000 [41,54-57,59-61]. High molecular weight proteins have been purified by precipitation using nonionic hydrophilic polymer, polyethylene glycol (PEG) [62,63]. Dextransucrase from *Streptococcus mutans* was purified by PEG precipitation [64] however there are only a few reports in the literature on *Leuconostoc mesenteroides* NRRL B-512F dextransucrase precipitation by PEG [31,32]. In the present study a simple method has been devised for obtaining the purified enzyme with appreciably higher specific activity and higher yields by fractionation using PEG 400 [59].

Extracellular dextransucrase has also been purified by phase-partition method [42,50]. Phase-partition occurs between dextran and polyethylene glycol (PEG). The addition of PEG solution to a dextran-rich aqueous solution, leads to the appearance of two phases; the top phase being rich in PEG while the bottom one is rich in dextran. The enzyme, dextransucrase preferentially goes into the dextran rich phase.

I.3.3 Properties of dextransucrase

The properties of dextransucrase have been extensively studied [40,49,55,56,60,61,65-69]. The enzyme exhibits maximum activity at a temperature of 30°C [40,55] and a pH of 5.2 [28,40,49,70]. The enzyme loses half of the activity on storing for two days at 4°C and loses 60% of the activity after 20 days at -15°C [49]. Calcium ions have been shown to affect the activity as well as the stability of dextransucrase [40,49,65]. The loss of activity could be prevented by adding calcium ions but the higher concentration of calcium acted as a competitive inhibitor [67]. The enzyme activity of dextransucrase is inhibited by EDTA and can be reversed by the addition of Ca^{2+} ions [49]. This shows the presence of Ca^{2+} in the naturally occurring enzyme.

Dextran is known to stabilize the enzyme against activity losses [61,65,69]. The dextran free enzyme loses its activity completely within a couple of hours [61]. The enzyme can be stabilized by various other agents [49,61,65]. The dextransucrase has been stabilized against activity losses by the addition of low concentrations of non-ionic polymers like dextran, PEG 20,000 or nonionic detergents like tween 80 and triton X100 [65]. The activity of dextransucrase is not inhibited by sulfhydryl reagents like N-ethyl maleimide and *p*-chloromercuribenzoate [55,71]. The enzyme is inhibited by

heavy metal ions [49,58], photo-oxidation [72] and also when exposed to radiation [66].

I.3.4 Amino acid composition of dextransucrase

The importance of amino acid analysis needs no emphasis. None of the remarkable advances of the last decade in protein and enzyme structure would have been possible without reliable techniques of amino acid determination. Modern techniques, which are largely chromatographic in nature, form an integral part of almost all biochemical structure investigations. Amino acid analysis bears a relationship to chemistry of proteins and peptides similar to that which elementary analysis bears to chemistry of simpler organic molecules. It gives an evidence as to the purity of proteins. When a protein of unknown composition is being analyzed, the key calculation is the correlation of its molecular weight with integral numbers of amino acid residues which are present in small molar proportions. Various methods like ion exchange [73,74], gas chromatography [75,76] and absorption spectroscopy [77,78] have been developed and used for amino acid determination.

The hydrolysis of a protein or polypeptide plays most important role in determining its amino acid content. The proteins are hydrolyzed by acid hydrolysis using hydrochloric acid (HCl), the most widely used agent [79-87], sulfonic acids

[88-90], alkaline hydrolysis using sodium hydroxide [91,92], enzymatic hydrolysis using pronase [83], aminopeptidases [84] thermolysin [93] and protease [94].

Not much is known about the amino acid composition of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. However, it has been reported for dextransucrase from *Streptococcus mutans* [85,86] and *Streptococcus sanguis* [87]. Striking feature of amino acid composition of these enzymes are that they are virtually devoid of cysteine and rich in acidic amino acid residues.

I.4 ACTIVE SITE MAPPING STUDIES OF DEXTRANSUCRASE BY CHEMICAL MODIFICATION

I.4.1 Mechanism of dextran synthesis

The mechanism of dextran formation by dextransucrase has been studied by several investigators [95-100]. Hehre [13] demonstrated for the first time, the synthesis of dextran using extracellular dextransucrase from *Leuconostoc mesenteroides*. It was further shown that unlike in other polysaccharide synthesis such as glycogen and starch, phosphorylated sugar intermediates were not involved in dextran synthesis [28].

It was reported that the enzyme requires two substrates, sucrose as a glucosyl donor and dextran as a glucosyl acceptor and catalyzes the glucosyl transfer reaction from sucrose to

acceptor dextran and produces dextran [95]. A mechanism was proposed in which, the donor (sucrose) and acceptor substrate (dextran) bind to distinct sites of the enzyme [95]. It was observed that dextransucrase catalyzes the hydrolysis of sucrose leading to the formation of glucosylated enzyme. This complex exhibits three competing activities, *viz.* i) hydrolysis where water molecules act as the acceptor, ii) acceptor reactions in the presence of other carbohydrates in addition to sucrose, iii) polymerization of glucose leading to dextran synthesis. The acceptor (ii) and polymerization reactions (iii), constitute to the transferase activity of the dextransucrase. The sucrase activity represents the reaction between the donor sucrose and a catalytic group and the transferase activity represents the reaction between acceptor dextran and a catalytic group which retains an intermediate form of the glucosyl residue [96]. This mechanism essentially requires dextran as primer for dextran synthesis.

A two-site insertion mechanism was also postulated for dextran synthesis using pulse and chase experiments with [^{14}C]sucrose [97]. It was shown that the dextran is biosynthesized by dextransucrase by the addition of glucose units to the reducing ends of growing chains. It was proposed that two nucleophiles at the active sites of dextransucrase attack two bound sucrose molecules to give two glucosyl units

covalently linked to the nucleophiles through C-1 [98]. It was postulated that in subsequent step, the nucleophilic attack of the C-6-OH of one of the glucosyl units onto C-1 of the other glucosyl intermediate results in the formation of α,(1⟶6) glucosidic bond. This releases one of the nucleophiles at the active site that attacks another sucrose molecule to give a new enzyme-glucosyl complex. The C-6-OH of this glucosyl unit then repeats the process of attacking C-1 of the dextran [98]. This process is continued between the two sites giving the synthesis of dextran by insertion of glucosyl units between the enzyme and the reducing end of growing chain. The polymerization is terminated when an acceptor molecule interacts with the active site of enzyme and one of its C-OH groups attacks the C-1 of the enzyme-glucosyl or dextranosyl complex and releases it from the enzyme. Dextran itself can act as an acceptor when one its C-3-OH group attacks C-1 centre forming a α,(1⟶3) bond. The proposed mechanism for dextran synthesis is shown in (Fig. I.1).

Carbohydrates such as glucose, fructose, maltose and dextran can also accept the glucosyl group from sucrose in the presence of dextransucrase which are termed as acceptor substrates [99]. It was shown by inhibition studies using deoxy-analogs of sucrose and maltose that the active site of dextransucrase has two equivalent sites for sucrose and one

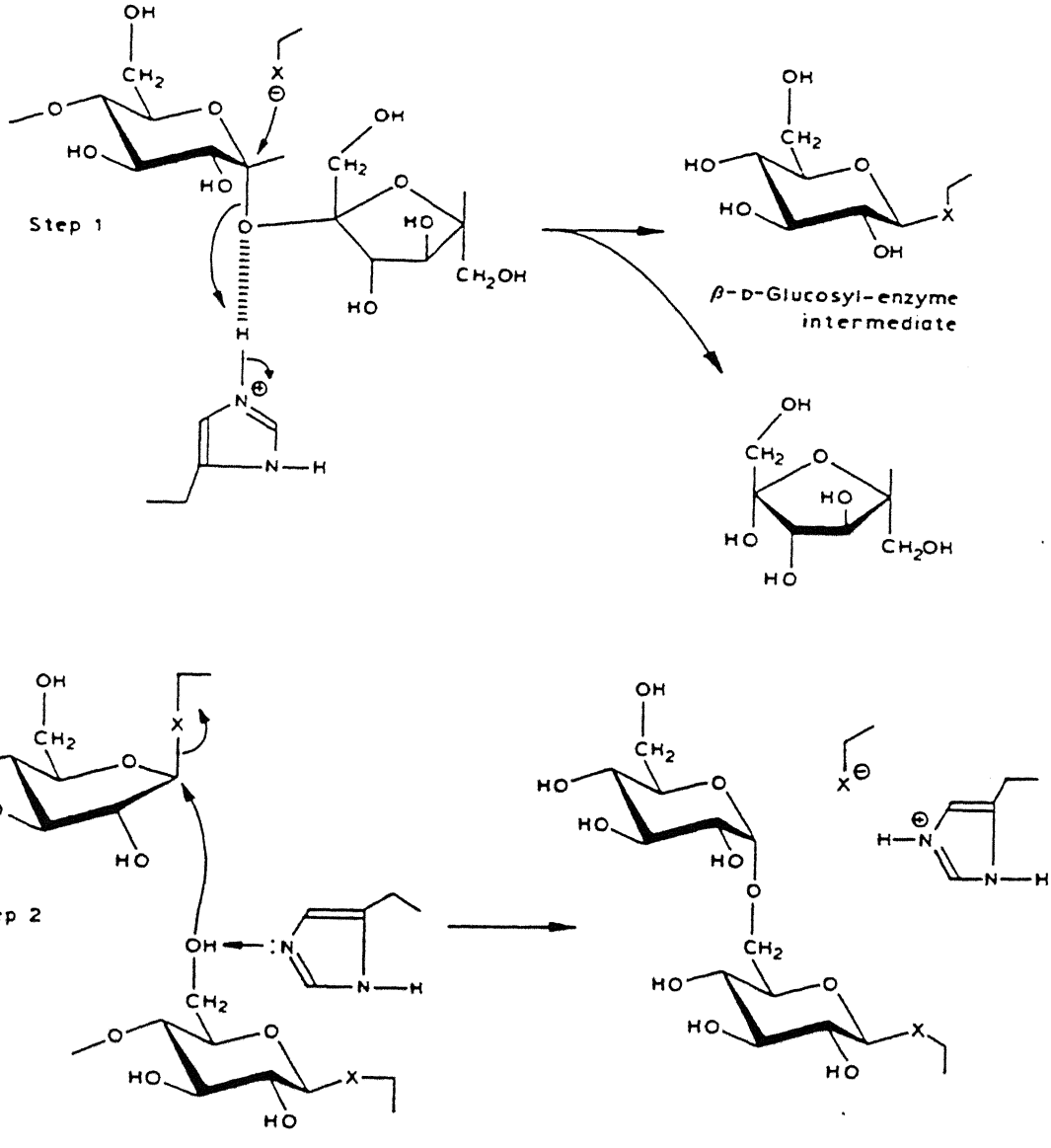


Figure I.1. Mechanism of synthesis of dextran by action of imidazole group of two histidine residues at the active site of dextransucrase. Step 1, Cleavage of sucrose by donation of a proton to D-fructose. Step 2, formation of α,(1⟶6) linkage by abstracting proton from the C-6-OH group, facilitating hydroxyl nucleophillic attack on C-1 of the D-glucosyl-enzyme intermediate.

site for acceptor binding [99]. Chemical modification studies with dextransucrase of *Leuconostoc mesenteroides* NRRL B-512F have shown that the two essential histidine residues are present at the active site [98]. Further, it was proposed in support to the two-site mechanism for dextran synthesis, that the imidazolium groups of these histidine residues donate protons to the leaving D-fructosyl moieties and the resulting imidazole groups facilitate the formation of $\alpha,(1\longrightarrow 6)$ glucosidic linkage by abstracting protons from the C-6-OH groups and become reprotonated for the next series of reactions [98]. It was shown that two sucrose binding sites and an acceptor binding site constitute the active site. It was further proposed that two sucrose sites are required for the synthesis of dextran and only one sucrose site for the synthesis of acceptor product. When one site is modified by chemical modification of one histidine residue, the synthesis of dextran stops, but synthesis of acceptor products can continue at the other site [100].

I.4.2 Active site of dextransucrase

Extensive work has been carried out on the mechanism of dextran synthesis and structural organization of active site of dextransucrase [95-100]. A dextran binding domain was isolated from both water soluble GTF-S [101] and water insoluble GTF-I

[102] extracellular glusosyltransferases from *Streptococcus sobrinus* by trypsin digestion of enzymes. It was further shown that the GTF-I is composed of 1600 amino acids and the enzyme can be divided in to three segments; (i) for sucrose splitting (1100 residues), (ii) for dextran binding (240 residues) and (iii) for unknown function (260 residues) from N-terminus [102]. For dextransucrases it has been proposed that the domain related substrate induced conformational changes are involved in catalysis. Kinetic studies have implicated substrate-induced conformational changes and sucrose binding increases enzyme affinity for dextran [101]. In addition, if sucrose binding is required to form the active catalytic site, then this accounts for the absence of a reverse reaction since sucrose is not present in the reverse reaction.

Less information is available on the nature of amino acids present at the active site. In a two-site mechanism for dextran synthesis it was shown that the two nucleophiles at the active site attack the two bound sucrose molecules to give two covalent intermediates [97]. Later, by chemical modification studies using diethyl pyrocarbonate it was shown that the two essential histidine residues are present at the active site. Further, it was proposed that the imidazolium groups of histidine residues donate protons to the leaving D-fructosyl moieties and the resulting imidazole groups facilitate the

formation of α-(1⟶6)-glucosidic linkage by abstracting protons from the C-6-OH groups [98]. It has been proposed that the two sucrose binding sites and an acceptor binding site constitute the active site [100].

An active site peptide containing covalently bound glucosyl group to aspartic acid has been isolated and sequenced from both GTF-S and GTF-I from *Streptococcus sobrinus* [103]. Further, a mechanism was also proposed in which a residue aspartic acid, stabilizes the carbonium ion in equilibrium with the covalent glucosyl-enzyme complex and another residue, histidine facilitates the departure of fructose by donating a proton to the glucoside oxygen. In the present study further elucidation of amino acid residues at the active site of dextransucrase has been carried out by chemical modification studies. It has been found by chemically modifying the enzyme by pyridoxal 5′-phosphate [104] and *o*-phthalaldehyde [105] that a lysine residue is essential for the activity of dextransucrase. These chemical modification methods are elaborated in the next section.

I.4.3 Chemical modification studies

Enzymes as biocatalysts catalyzing the biochemical reactions in the living organisms as well as their use in biochemical processes has stimulated the investigations for the

understanding of their structural organization and mechanism of action. The characteristic feature of enzymes is their ability to catalyze a reaction at a very high rate under mild conditions. Several approaches have been used to understand the enzyme mechanism. They are reaction kinetics [106,107], chemical modification [108-111], site-directed mutagenesis [112-114], X-ray diffraction [115,116], nuclear magnetic resonance [117,118] and monoclonal antibodies [119,120]. By studying the reaction kinetics one can learn about the order in which substrates bind and products are released from the enzyme. However, information regarding the molecular details of mechanism by which these transformations take place and how the structural elements contribute to the process are not obtained. By the combination of reaction kinetics with chemical modification technique a great deal of information regarding the structural and functional aspects of the enzyme can be obtained.

Chemical modification is one of the most versatile techniques for identification of functional groups of proteins and enzymes. Modification of enzyme involves binding of a chemical moiety to the side chain(s) of amino acid residues in the enzyme which induces some measurable change in property of the enzyme. Of the 20 natural amino acids only those possessing a polar side chain are normally the targets for chemical

modification. A specific chemical modification results in the quantitative binding of the functional group belonging to a unique amino acid residue without affecting other functional groups or the conformation of the enzyme molecule.

I.4.3.1 *The active site structure*

The active site of an enzyme is the region having a particular arrangement of the functional groups that are involved in catalyzing the specific reaction. The primary structure of the enzyme folds in such a way so as to create a region that has correct molecular dimensions, appropriate topology and optimal alignment of functional groups and hydrophobic regions to accommodate the specific substrate. To decipher the catalytic mechanism of an enzyme it is essential to know the structural elements (functional group of amino acids) present and the three dimensional conformation of site.

I.4.3.2 *Specificity of enzymes*

Enzymes are very specific with respect to their substrates compared to chemical catalysts; that is, enzymatic reactions rarely have side products. The noncovalent forces through which substrates and other molecules bind to the enzyme are identical to the forces that dictate the conformation of the enzymes which involve Van der Waals forces, hydrogen bonding,

electrostatic, and hydrophobic interactions [121]. It is well established that amino acid residues forming the binding site are arranged in a particular fashion, which allows only the substrate molecules to interact in a specific manner [121].

I.4.3.3 *Essential amino acid residues and their behavior*

The functional side chains of amino acids located at or near the active site are involved in the substrate binding and/or catalysis. These amino acid residues are thus crucial for the enzyme activity and the modification of their functional groups leads to the loss of catalytic activity. The amino acid residues possessing functional side chains which participate in the enzyme catalysis are called essential amino acid residues. Usually amino acids containing reactive side chains or functional groups are located at the active site of enzymes. These include acidic amino acids [glutamate and aspartate], basic amino acids [lysine, arginine and histidine], polar uncharged amino acids [serine, cysteine and tyrosine] and side chains of methionine [possessing a nucleophilic sulfur] and tryptophan [having heterocyclic indole side chain] [122]. These residues are present in a specific arrangement so that the substrate molecules are able to interact and form enzyme-substrate complexes.

Several factors are responsible for different behavior of active site residues. Some of the important factors are polarity which affects the dissociable side chains, hydrogen bonding effects, which may stabilize the neutral or ionic species; electrostatic effects (presence of charges in the vicinity of the group); and the steric effects by the other side chains [123]. All these factors differentiate the essential amino acid residues from non essential ones.

I.4.3.4 *Stoichiometry of reaction*

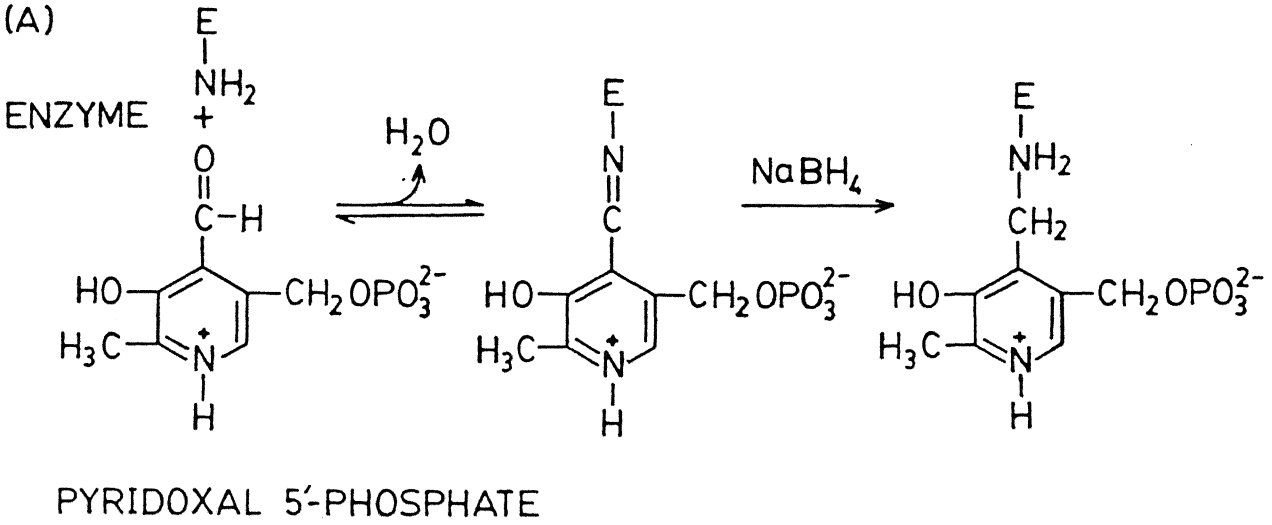
Stoichiometry of reaction is one of the most important property of chemical modification by which the number of essential residues involved in the catalytic reaction of enzyme can be determined. Different approaches such as radioactive labelling [124], amino acid analysis [125], and spectroscopy [126,127] have been used to determine the stoichiometry. Of these, spectroscopic technique is the most convenient for determining the incorporation of spectrally active group. The incorporation of the modifier in the enzyme can be correlated with residual activity of the enzyme. The number of amino acid residues critical for the enzyme activity thus can be determined.

I.4.3.5 *Group specific reagents*

These modifiers belong to a class which bind covalently with amino acid side chains. Many reagents have been developed for the modification of different amino acid side chains [128,129]. The specific binding of these chemical modifiers results either from enhanced reactivity of residues due to their local environment in the native protein or due to the specificity of the particular reagent to a site in the protein or perhaps from the combination of both effects. Reagents and reaction conditions for the modification of protein are chosen depending upon the properties of the protein and the purpose of investigation. The essential residues involved in the catalysis can be selectively modified by a variety of reagents.

I.4.3.5.1 Lysine specific reagents

Lysine specific reagents include pyridoxal 5′-phosphate (PLP) and 2,4,6-trinitrophenyl-1-sulphonic acid (TNBS). These chemical modifiers are highly selective in nature. PLP has been used extensively to study the role of the essential lysine residues in the enzyme catalysis [130-134]. Reaction of lysine residue with PLP results in Schiff's base formation (Fig. I.2.A). Subsequent reduction of the Schiff's base with sodium borohydride gives an irreversible derivative N^{ε}-phosphopyridoxyllysine. The group introduced, in the



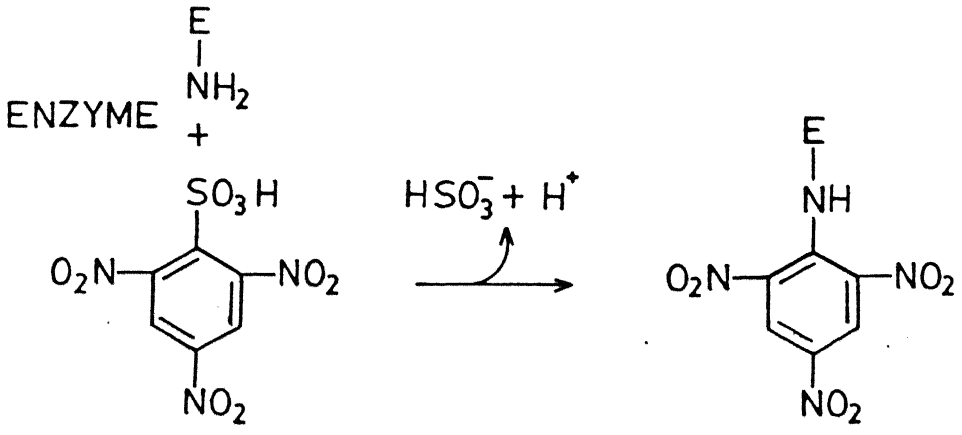


Figure I.2. Modification of lysine by specific reagents

reaction product is a chromophore with an extinction coefficient of 9700 $M^{-1}cm^{-1}$ at 325 nm (Table I.2), which allows quantitation. The chromophore is also fluorescent, and fluorescence emission spectra on excitation at wave length 325 nm gives a characteristic maxima centered near 400 nm depending upon the nature of active site of the enzyme.

2,4,6-Trinitrobenzene-1-sulphonic acid specifically reacts with amino group of lysine and has been used for chemical modification studies of various enzymes [135,136]. It reacts with ε-amino group of lysine to give a trinitrophenyl derivative (Fig. I.2.B). This has an absorption maximum centered around 367 nm [129], and TNBS modification can be quantitated via absorbance measurements at this wave length. The extinction coefficient of the derivative is 11,000 $M^{-1}cm^{-1}$ (Table I.2). As with many of the other reagents, the reaction of TNBS with sulfhydryl groups can also occur, although this problem has not been frequently reported.

PLP usually binds reversibly to the enzyme [137-139] but in malate dehydrogenase the binding of PLP has been shown irreversible [140], whereas, TNBS binds only irreversibly. This characteristic feature is useful in predicting the nature of active site. The reversibility is also dependent on nature and orientation of the catalytic amino acid residues present at the active site of the enzymes.

Table I.2

Reagent	Absorption maximum (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)
1. Pyridoxal 5′-phosphate [129,132]	325	8,500
2. 2,4,6 Trinitrobenzene-1-sulphonic acid [129]	367	11,000
3. *p*-Chloromercuricphenyl-sulphonic acid [129]	250	7,500
4. N-Ethyl maleimide [129]	300	620
5. 5, 5′-Dithiobis (2-nitro-benzoic acid) [78]	412	13,600
6. *o*-Phthalaldehyde [144-146]	337	7,660

I.4.3.5.2 Cysteine specific reagents

A wide variety of reagents are available for the modification of sulfhydryl group of cysteine. Few reagents like p-chloromercuricphenyl sulfonic acid (PCMS) (Fig. I.3.A), N-ethyl maleimide (NEM) (Fig. I.3.B), 5, 5′-dithiobis(2-nitro-benzoic) acid (DTNB) (Fig. I.3.C) are highly selective in nature and have been used in studying the role of essential cysteine residues in the enzyme catalysis.

The mercury based reagent PCMS is the most specific for sulfhydyl groups. The *p*-mercuribenzoate derivative of a sulfhydryl has an extinction coefficient of 7500 $M^{-1}cm^{-1}$ at

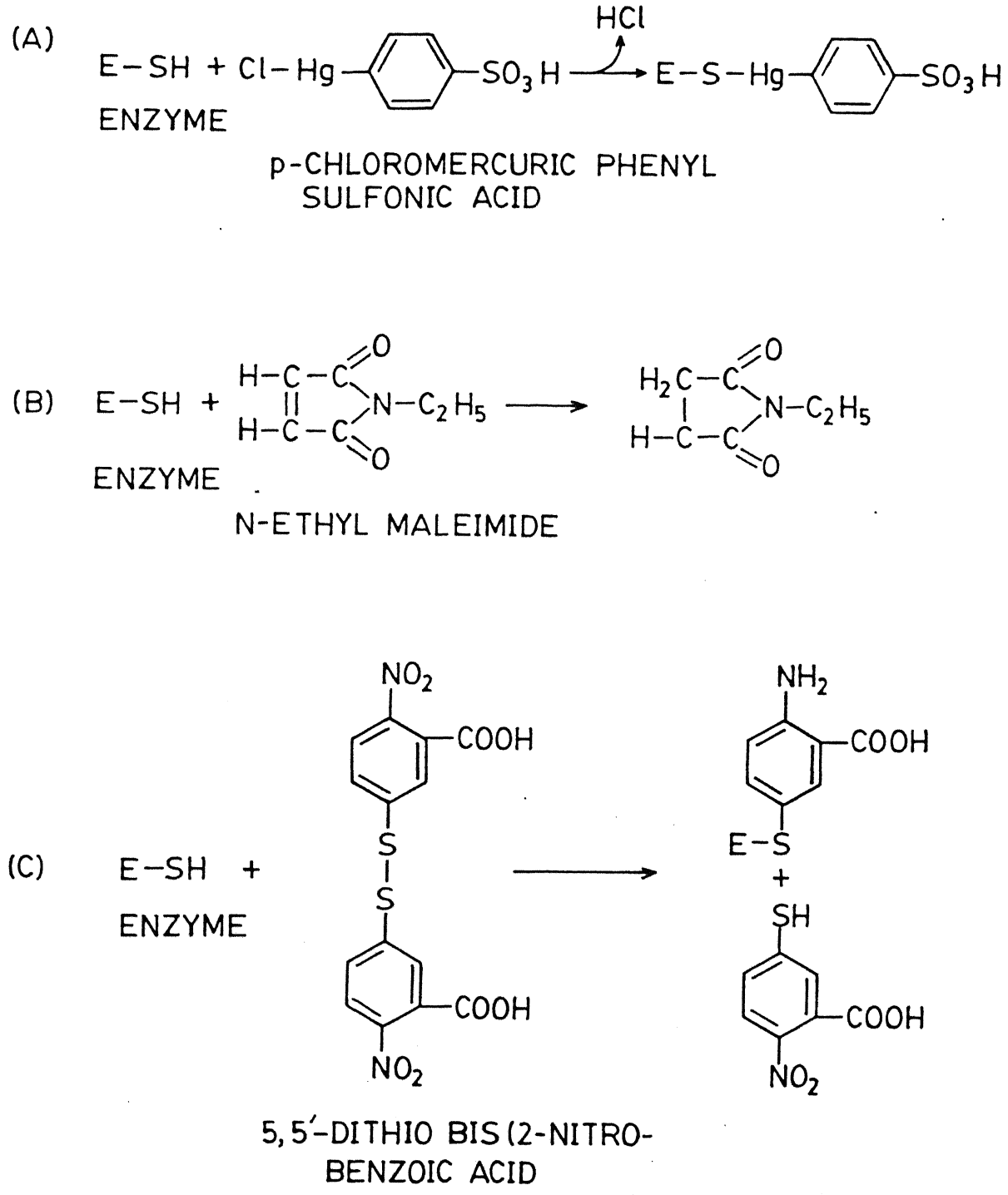


Figure I.3. Modification of cysteine by specific reagents

250 nm of (Table I.2). NEM reacts with sulfhydryls to give acid-stable derivatives. Reactions with other groups may occur, but these derivatives tend to be acid labile. The derivative produced has an absorption maximum at 300 nm, with an extinction coefficient of 600 $M^{-1}cm^{-1}$ (Table I.2), which is too low to allow reasonable quantitation except at high protein concentrations or high degree of modification.

The reagent that is widely used with sulfhydryls is DTNB. This forms a mixed disulfide with cysteine and releases the thionitrobenzoate anion (Fig. I.3.C), which can be quantitated spectrophotometrically by absorbance measurements at 412 nm and has an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (Table I.2). This coefficient allows easy and rapid titration of sulfyhdryl groups in native or denatured proteins.

I.4.3.6 Kinetic analysis of modification

Kinetic investigations of the modification reaction with enzyme can give useful information regarding the mechanism of inactivation process [106,141-143]. The kinetic mechanism of chemical modification reaction depends on the conditions of the reaction system. Usually, the inactivation process follows pseudo-first order kinetics (*i.e.* the concentration of modifier is much higher than the concentration of the enzyme) [122].

The observed first order rate constant (k_{obs}) can be calculated from the following equation.

$$-\ln\left[\frac{[E]_t}{[E]_o}\right] = k_{obs}\ t$$

Where $[E]_o$ and $[E]_t$ represent the enzyme activities at zero and at any given time t, respectively. Plot of pseudo-first order rate constants against inhibitor concentration gives a straight line. The slope of this plot yields second order rate constant.

I.4.3.7 Use of o-phthalaldehyde, a fluorogenic bifunctional reagent in chemical modification studies

Fluorometry has proven to be a valuable technique in the study of the active site of enzymes [144-153]. A specific chemical modifier forms a fluorescent adduct with the functional group of enzyme, accompanied by the loss of catalytic activity. It is essential that fluorescent adduct should have absorption and fluorescence properties distinct from that of tyrosyl and trypytophanyl residues of the protein. Fluorescence characteristics can also be helpful in understanding the nature of the catalytic environment because the maxima of the emission spectra is red or blue shifted

depending on the characteristic environment present at the active site.

A fluorogenic bifunctional reagent, *o*-phthalaldehyde is amongst very few compounds which have been used for the characterization of the nature of the active site of the enzyme. The fluorescence property of this compound on reacting with amino acid in presence of reducing agents like β-mercaptoethanol was first reported by Roth [154]. Benson and Hare [155] identified the product formed by the reaction of *o*-phthalaldehyde with amino acid and β-mercaptoethanol to be an isoindole adduct having a characteristic fluorescence emission pattern. It was also observed that *o*-phthalaldehyde forms a non fluorescent adduct with amino groups in the absence of β-mercaptoethanol [156,157]. The use of *o*-phthalaldehyde as an active site reagent was first reported by Palczewski *et al.* [158]. They showed that aldolase can be inactivated when treated with *o*-phthalaldehyde. It forms an isoindole derivative by cross linking, ε-amino group of lysine and sulfhydryl group of cysteine. The mechanism of isoindole formation is shown in Fig. I.4.

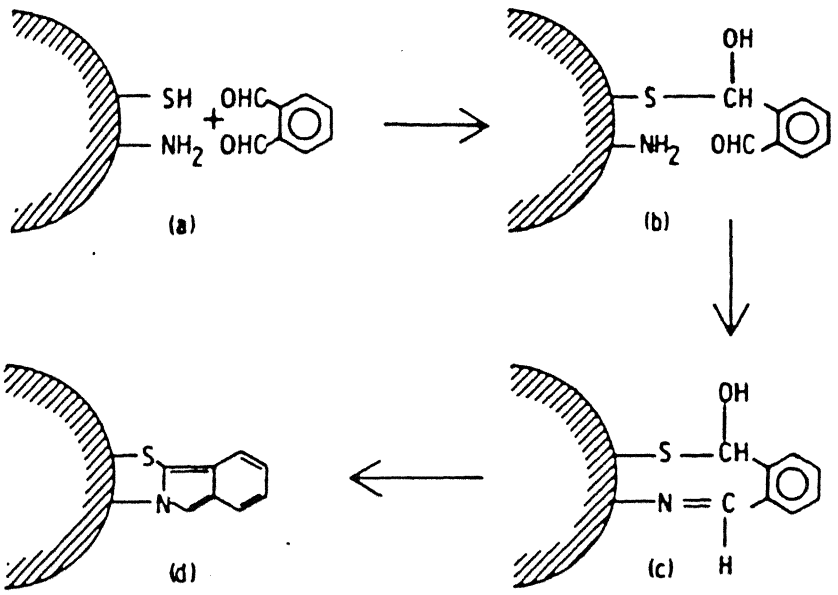


Figure I.4. Schematic representation of the reaction of o-phthalaldehyde with sulfhydryl group of cysteine and ε-amino group of lysine residues of an enzyme.

The reaction of *o*-phthalaldehyde with enzymes is characterized by the absorbance maximum at 337 nm and fluorescence emission maximum in the range 400-450 nm [144-146,158-163]. Free *o*-phthalaldehyde does not exhibit any fluorescence over the spectral range 400-500 nm upon excitation at 337 nm [145]. The variation in the fluorescence emission maximum arises due to difference in the micro environment of cysteine and lysine residues participating in the formation of isoindole derivative.

The molar transition energy (E_T) is calculated from the fluorescence emission maximum (λ_{em}) of an isoindole derivative using following relationship [158].

$$E_T = 2.985\ \lambda_{em} - 1087.28$$

Plot of λ_{em} (nm) and E_T according to above equation is shown in Fig. I.5. Minimum transition energy value of 121 kJ/mol was obtained for 1-(β-Hydroxyethylthio)-2-β-hydroxy-ethyl-isoindole (EA adduct), a synthetic isoindole, in non polar medium of hexane. Maximum E_T was obtained in H_2O, which was more than 250 kJ/mol. On comparing the molar transition energies of synthetic isoindole EA, in various solvents and the isoindole derivative formed with cysteine and lysine residues of enzymes, it has been shown that active site of enzymes are relatively hydrophobic in nature. The values of E_T obtained for enzyme-*o*-phthalaldehyde adduct were close to synthetic isoindole in hexane which indicated the relatively hydrophobic nature at the catalytic center. *o*-Phthalaldehyde, besides acting as an active site specific reagent for many enzymes [145,146,160-163], can also bind at the non catalytic site of enzyme leading to conformational changes and resulting in inactivation [151].

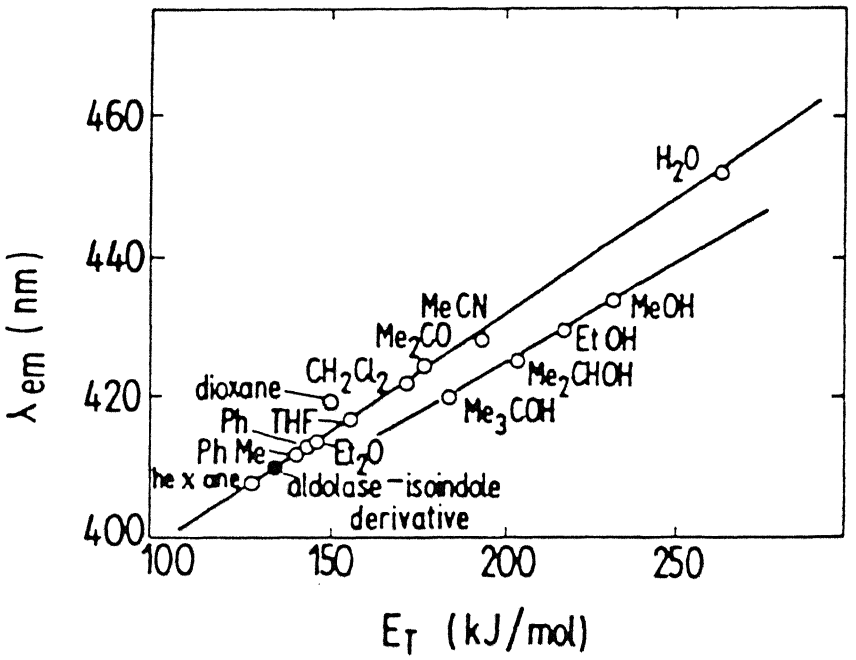


Figure I.5. Relationship between λ_{max} fluorescence of a synthetic isoindole, 1-[(β-hydroxyethyl)thiol]-2-(β-hydroxyethyl) isoindole, an (EA) adduct and molar transition energies (E_T) in different solvents. Fluorescence maxima of 8 μM EA adduct in: *n*-hexane, toluene (PhMe), benzene (Ph), ethyl ether, (Et_2O), 1,4-dioxane (dioxan), tetrahydrofuran (THF), methyl chloride (CH_2Cl_2), acetone (Me_2CO), acetonitrile (MeCN), *t*-butanol (Me_3COH), *iso*-propyl alcohol (Me_2CO), ethyl alcohol (EtOH), methyl alcohol (MeOH) and water (H_2O) [156].

I.5 OBJECTIVES OF THE PRESENT STUDY

Leuconostoc mesenteroides NRRL B-512F is known to synthesize enzyme dextransucrase when grown in sucrose medium. The enzyme catalyzes the formation of dextran from sucrose. Although, various media for the maintenance of *Leuconostoc mesenteroides* have been reported but none of them suggests that the enzyme production by the *Leuconostoc mesenteroides* NRRL B-512F is influenced by the medium, in which it is maintained. In the present study, an attempt was made to screen a variety of media and select a medium for culture maintenance that is capable of inducing higher production of dextransucrase and to ascertain the extent, to which the yield of dextransucrase is regulated by the medium used for maintaining the cultures of *Leuconostoc mesenteroides* NRRL B-512F.

A series of reports are available on the production of dextransucrase under different culture conditions. The temperatures in the range 20 to 30°C have been used for dextransucrase production. Our interest was to establish the extent of the temperature effect on the dextransucrase production within a close range. There are conflicting reports on the production of dextransucrase using shaken and static flask cultures. It has been demonstrated that the aeration and the higher agitation rates of culture media do not favor the production of dextran as well as the dextransucrase, though mild aeration and

agitation have been used with uncontrolled pH in the batch process. This led us to re-investigate and compare the enzyme production in still and shaken flask cultures. Different combinations of temperature, pH and sucrose concentration have been used for maximum activity of dextransucrase. The present study re-defines certain optimal conditions for dextransucrase production.

Various media compositions have been employed for production and improvising the yield of dextransucrase. Only a few reports elaborate on the effect of an individual or an additional nutrient. A thorough study was carried out to study the effect of certain nutrients and salts on the enzyme production.

Dextransucrase has been purified by various techniques involving salt precipitation, ultrafiltration and chromatograhpic methods. Dextransucrase exists in an aggregated form in the presence of dextran resulting in high molecular weight. High molecular weight proteins have been purified by precipitation using the nonionic hydrophilic polymer, polyethylene glycol (PEG). In the present study a simple and effective method was devised for purification of dextransucrase by fractionation with PEG of different molecular weights.

Dextransucrase has also been purified by phase-partition method. Phase-partition occurs between dextran and polyethylene glycol (PEG). The addition of PEG solution to a dextran-rich aqueous solution, leads to the appearance of two phases; the top phase being rich in PEG while the bottom one is rich in dextran. The enzyme was purified by phase-partitioning, using PEG of different molecular weights.

Dextransucrase can be stabilized against activity losses by various agents. In the present study, effects of alternative stabilizers and temperature conditions for storage of dextransucrase were studied.

Although extensive work has been carried out on the mechanism of dextran synthesis and structural organization of catalytic site of dextransucrase, not much information is available on the nature of amino acids at the active site. By chemical modification studies it was shown earlier that the two essential histidine residues are present at the active site. In the present study it has been shown for the first time, that an essential lysine residue is present at the active site chemically modifying the dextransucrase with pyridoxal 5′-phosphate (PLP) and 2,4,6-trinitrobenzenesulphonic acid (TNBS), the lysine-specific reagents and a fluorogenic bifunctional reagent, *o*-phthalaldehyde.

REFERENCES

1. Pasteur, L. (1861) Bull. Soc. Chim. Paris, 30.
2. Scheibler, C. (1874) Z. Ver. Dtsch. Zucker Ind., 24, 309.
3. Van Tieghem, P. (1878) Ann. Sci. Nat. Bot. Biol. Veg., 7, 180-203.
4. Van Cleve, T.W., Schaefer, W.C. and Rist, C.E. (1956) J. Am. Chem. Soc., 78, 4435-4438.
5. Krasse, B. (1965) Arch. Oral. Biol., 10, 223.
6. Banghart, S.B. (1967) Arch. Oral. Biol., 12, 11.
7. Jeanes, A., Haynes, W.C., Wilham, C.A., Rankin, J.C., Melvin, E.H., Austin, M.J., Cluskey, J.E., Fisher, B.E., Tsuchiya, H.M. and Rist, C.E. (1954) J. Am. Chem. Soc., 76, 5041-5052.
8. Tsuchiya, H.M., Hellman, N.N., Koepsell, H.J., Corman, J., Stringer, C.S., Rogovin, S.P., Bogard, M.O., Bryant, G., Feger, V.H., Hoffman, C.A., Senti, F.R. and Jackson, R.W. (1955) J. Am. Chem. Soc., 77, 2412-2419.
9. Castillo, E., Iturbe, F., Lopez-Munguia, Pelenc, V., Paul, F. and Monsan, P. (1992) Ann. N. Y. Acad. Sci., 672, 425-430.
10. Evans, T.H. and Hibbort, H. (1946) Adv. Carbohydr. Chem., 2, 202-203.
11. Hehre, E.J. (1951) Adv. Enzymol., 11, 297-337.

12. Ebert, K.H. and Schenk, G. (1968) Adv. Enzymol., 30, 179-221.
13. Hehre, E.J. (1941) Science, 93, 237-238.
14. Stacey, M. and Youd, F.R. (1938) Biochem. J., 32, 1943-1945.
15. Stacey, M. (1942) Nature, 149, 639.
16. Hehre, E.J. (1946) J. Biol. Chem., 163, 221-223.
17. Jeanes, A., Wilham, C.A. and Miers, J.C. (1948) J. Biol. Chem., 176, 603-615.
18. Hehre, E.J. (1955) Methods Enzymol., 1, 178-184.
19. Jeanes, A., Wilham, C.A., Tsuchiya, H.M. and Haynes, W.C. (1957) Arch. Biochem. Biophys., 71, 293-302.
20. Jeanes, A. (1965) Methods Carbohydr. Chem., 5, 118-126.
21. Jeanes, A. (1965) Methods Carbohydr. Chem., 5, 127-132.
22. Lawford, G.R., Kligerman, A., Williams, T. and Lawford, H.G. (1979) Biotechnol. Bioeng., 21, 1121-1131.
23. Landon, R.S. and Webb, C. (1990) Process Biochem., 25, 19-23.
24. Lee, S., Choi, S., Kim, E. and Koo, Y. (1992) Biotechnol. Lett., 14, 379-384.
25. Lazic, M.L., Veljkovic, V.B., Vucetic, J.I. and Vrvic, M.M. (1993) Enzyme Microb. Technol., 115, 334-338.
26. Ajongwen, W.J. and Barker, P.E. (1993) J. Chem. Technol. Biotechnol., 56, 113-118.

27. Schwartz, R.D. and Bodie, E.A. (1984) App. Env. Microbiol., 48, 678-679.

28. Alsop, R.M. (1983) Prog. Ind. Microbiol., 18, 1-44.

29. Koepsell, H.J. and Tsuchiya, H.M. (1952) J. Bacteriol., 63, 293-295.

30. Brown, D.E. and McAvoy, A.J. (1990) J. Chem. Technol. Biotechnol., 48, 405-414.

31. Barker, P.E., Zafar, I. and Aslop, R.M. (1987) Separations for Biotechnology, pp 127-151, ed. Verrall, M.S. and Hudson, M.J., Ellis Horwood, Chichester, UK.

32. Ajongwen, J., Akintoye, A., Barker, P.E., Ganetsos, G., and Shieh, M.T. (1993) Chem. Eng. J. 51, B43-B50.

33. Barker, P.E., Ganetsos, G., Ajongwen, J. and Akintoye, A. (1992) Chem. Eng. J., 50, B23-B28.

34. Barker, P.E., Ganetsos, G. and Ajongwen, J. (1993) J. Chem. Technol. Biotechnol., 57, 21-26.

35. El-Sayed, A.H.M.M., Abdul-Wahid, K. and Coughlin, R.W. (1992) Biotechnol. Bioeng., 40, 617-624.

36. El-Sayed, A.H.M.M., Mahmoud, W.M. and Coughlin, R.W. (1990) Biotechnol. Bioeng., 36, 83-91.

37. El-Sayed, A.H.M.M., Mahmoud, W.M. and Coughlin, R.W. (1990) Biotechnol. Bioeng., 36, 346-353.

38. El-Sayed, A.H.M.M., Mahmoud, W.M. and Coughlin, R.W. (1990) Biotechnol. Bioeng., 36, 338-345.

39. Lopez, A. and Monsan, P. (1980) Biochimie, 62, 323-329.

40. Kaboli, H. and Reilly, P.J. (1980) Biotechnol. Bioeng., 22, 1055-1069.

41. Monsan, P. and Lopez, A. (1981) Biotechnol. Bioeng., 23, 2027-2037.

42. Monsan, P., Paul, F., Auriol, D. and Lopez, A. (1987) Methods Enzymol., 136, 239-254.

43. Robyt, J.F. (1986) Dextran. In: Encyclopedia of Polymer Science and Technology, vol 4. John Wiley & Sons, pp 752-767.

44. Ito, M., Shigata, S. and Sato, M. (1988) Jpn. Kokai Tokkyo Koho JP 63,253,026 4pp.

45. DeMan, J.C., Rogosa, M. and Sharpe, M.E. (1960) J. Appl. Bacteriol., 23, 130-135.

46. Bhatnagar, R., Bhatnagar, N. and Prabhu, K.A. (1985) IRCS Med. Sci., 13, 129-130.

47. Tsuchiya, H.M., Koepsell, H.J., Corman, J., Bryant, G., Bogard, M.O., Feger, V.H. and Jackson, R.W. (1952) J. Bacteriol. 64, 521-526.

48. Neely, W.B. and Nott, J. (1962) Biochemistry, 1, 1136-1140.

49. Robyt, J.F. and Walseth, T.F. (1979) Carbohydr. Res., 68, 95-111.

50. Paul, F., Auriol., D., Oriol, E. and Monsan, P. (1984)

Ann. N. Y. Acad. Sci., 434, 267-270.

51. Barker, P.E. and Ajongwen, N.J. (1991) Biotechnol. Bioeng., 37, 703-707.
52. Lazic, M.L., Veljkovic, V.B., Savic, D.S., Rutic, D.J., Vucetic, J.I. and Vrivic, M.M. (1991) World J. Microbiol. Biotechnol., 7, 25-28.
53. Veljkovic, V.B., Lazic, M.L., Rutic, D.J., Jovanoic, S.M. and Skala, D.U. (1992) Enzyme Microb. Technol., 14, 665-668.
54. Pennell, R.D. and Barker, P.E. (1992) J. Chem. Technol. Biotechnol., 53, 21-27.
55. Kobayashi, M. and Matsuda, K. (1980) Biochim. Biophys. Acta, 614, 46-62.
56. Miller, A.W., Eklund, S.H. and Robyt, J.F. (1986) Carbohydr. Res., 147, 119-133.
57. Fu, D. and Robyt, J.F. (1990) Prep. Biochem., 20, 93-106.
58. Rhee, S.H. and Lee, C.-H. (1991) J. Microbiol. Biotechnol., 1, 176-181.
59. Goyal, A. and Katiyar, S.S. (1994) J. Microbiol. Methods, 20, 225-231.
60. Kobayashi, M. and Matsuda, K. (1986) J. Biochem., 100, 615-621.
61. Willemot, R.M., Monsan, P. and Durand, G. (1988) Ann. N.Y. Acad. Sci., 542, 169-172.

62. Honig, W. and Kula, M.R. (1976) Anal. Biochem., 72, 502-512.

63. Meikka, S.I. and Ingham, K.C. (1978) Arch. Biochem. Biophys., 191, 525-536.

64. Russell, R.R.B. (1979) FEMS Microbiol. Lett., 6, 197-199.

65. Miller, A.W. and Robyt J.F. (1984) Biochim. Biophys. Acta, 785, 89-96.

66. Miller, A.W. and Robyt, J.F. (1986) Biochim. Biophys. Acta, 870, 198-203.

67. Miller, A.W. and Robyt, J.F. (1986) Biochim. Biophys. Acta, 880, 32-39.

68. Kobayashi, M., Mihara, K. and Matsuda, K. (1986) Agric. Biol. Chem., 50, 551-556.

69. Kobayashi, M., Yokoyama, I. and Matsuda, K. (1986) Agric. Biol. Chem., 48, 221-223.

70. Neely, W.B. (1958) J. Am. Chem. Soc., 80, 2010-2013.

71. Kobayashi, M., Yokoyama, I. and Matsuda, K. (1985) Agric. Biol. Chem., 49, 3189-3195.

72. Miller, A.W. and Robyt, J.F. (1986) Arch. Biochem. Biophys., 248, 779-586.

73. Moore, S. and Stein, W.H. (1954) J. Biol. Chem., 211, 893-906.

74. Hamilton, P.B. (1966) Advan. Chromatog., 2, 3.

75. Lamkin, W.M., and Gehrke, C.W. (1965) Anal. Chem., 37, 383-389.

76. Darbre, A. and Blau, K. (1965) J. Chromatogr., 17, 31-49.

77. Goodwin, T.W. and Morton, R.A. (1946) Biochem. J., 40, 628-632.

78. Ellman, G.L. (1959) Arch. Biochem. Biophys., 82, 70-77.

79. Hirs, C.H.W. (1956) J. Biol. Chem., 219, 611-621.

80. Moore, S. and Stein, W.H. (1963) Methods Enzymol., 6, 819-831.

81. Moore, S. (1963) J. Biol. Chem., 238, 235-237.

82. Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem., 238, 622-627.

83. Sletten, K., Dus, K., De Klerk, H. and Kamen, M.D. (1968) J. Biol. Chem., 243, 5492-5506.

84. Salnikow, J., Liao, T.-H., Moore, S. and Stein, W.H. (1973) J. Biol. Chem., 248, 1480-1488.

85. Shimamura, A., Tsumori, H. and Mukasa, H. (1982) Biochim. Biophys. Acta, 702, 72-80.

86. Mukasa, H., Shimamura, A. and Tsumori, H. (1989) J. Gen. Microbiol., 135, 2055-2063.

87. Grahame, D.A. and Mayer, R.M. (1985) Carbohydr. Res., 142, 285-298.

88. Liu, T.-Y. and Chang, Y.H. (1971) J. Biol. Chem., 246, 2842-2848.

89. Niece, R.L. (1975) J. Chromatogr., 103, 25-32.

90. Penke, B., Ferenczi, R. and Kovacs, K. (1974) Anal. Biochem., 60, 45-50.

91. Noltman, E.A., Mahowald, T.A. and Kuby, S.A. (1962) J. Biol. Chem., 237, 1146-1154.

92. Hugli, T.E. and Moore, S. (1972) J. Biol. Chem., 247, 2828-2834.

93. Matsubara, H. and Sasaki, R.M. (1968) J. Biol. Chem., 243, 1732-1757.

94. Satake, K., Sasakawa, S. and Maruyama, T. (1963) J. Biochem. (Tokyo), 53, 516-517.

95. Kobayashi, M., Yokoyama, I. and Matsuda, K. (1986) Agric. Biol. Chem., 50, 2585-2590.

96. Parnaik, V.K., Luzio, G.A., Grahame, D.A., Ditson, S.L. and Mayer, R.M. (1983) Carbohydr. Res., 121, 257-268.

97. Robyt, J.F., Kimble, B.K. and Walseth, T.F. (1974) Arch. Biochem. Biophys., 165, 634-640.

98. Fu, D. and Robyt, J.F. (1988) Carbohydr. Res., 183, 97-109.

99. Tanriseven, A. and Robyt, J.F. (1992) Carbohydr. Res., 225, 321-329.

100. Su, D. and Robyt, J.F. (1994) Arch. Biochem. Biosphys., 308, 471-476.

101. Mooser, G. and Wong, C. (1988) Infect. Immun., 56, 880-884.

102. Abo, H., Matsumura, T, Kamada, T., Ohta, H., Fukui, K., Kato, K. and Kagawa, H. (1991) J. Bacteriol., 173, 989-996.

103. Mooser, G., Hefta, S.A., Paxton, R.J., Shively, J.E. and Lee, T.D. (1991) J. Biol. Chem., 266, 8916-8922.

104. Goyal, A. and Katiyar, S.S. (1994) J. Enz. Inhibition (in press).

105. Goyal, A. and Katiyar, S.S. (1994) Biochem. & Molec. Biol. Int. (in press).

106. Strickland, S. and Massey, V.S. (1973) J. Biol. Chem., 248, 2953-2962.

107. Jabalquinto, A.M., Eyzaguirre, J. and Cardemil, E. (1983) Arch. Biochem. Biophys., 225, 338-343.

108. Anderson, C. and Morgenstern, R. (1990) Biochem. J., 272, 479-484.

109. Creighton, T.E. (1989) Protein Structure: A practical approach, pp145-153 IRL press, Oxford.

110. Dugan, R.E. and Katiyar, S.S. (1986) Biochem. Biophys. Res. Commun., 141, 278-284.

111. Puri, R.N., Porter, J.W. and Katiyar, S.S. (1982) Biochem. Biophys. Acta, 713, 29-38.

112. Wagner, C.R. and Benkovic, S.J. (1990) Trends Biotechnol.,

8, 263-270.

113. Shaw, W.V. (1987) Biochem. J., 246, 1-17.

114. Knowles, J.R. (1987) Science, 236, 1252-1258.

115. Carter, P. and Well, J.A. (1988) Nature, 332, 564-568.

116. Hajdu, J. and Johnson, L.N. (1990) Biochemistry, 29, 1669-1678.

117. Wuthrich, K.J. (1989) Acc. Chem. Res., 22, 36-44.

118. Wuthrich, K., Wider, G., Wagner, G. and Braun, W. (1982) J. Mol. Biol., 155, 311-319.

119. Polakis, P.G. and Wilson, J.E. (1985) Arch. Biochem. Biophys., 236, 328-337.

120. Ureta, J., Smith, A.D. and Wilson, J.E. (1986) Arch. Biochem. Biophys., 246, 419-427.

121. Voit, D. and Voit, J.G. (1990) Biochemistry, John Wiley and Sons, New York.

122. Eyzaguirre, J. (1987) Chemical Modification of Enzymes: Active site studies, John Wiley and Sons, New York.

123. Cohen, L.A. (1970) The enzymes, 3rd ed. pp 147-211.

124. Katusz, R.M. and Colman, R.F. (1991) Biochemistry, 30, 11230-11238.

125. Rohbach, M.S. and Bodely, J.W. (1977) Biochemistry, 16, 1360-1363.

126. Bazaes, S., Beytia, E., Jabalquinto, A.M., Solis de Ovando, F., Gomez, I. and Eyzaguirre, J. (1980)

138. Katiyar, S.S. and Porter, J.W. (1982) Biochem. Biophys. Res. Commun., 107, 1219-1223.

139. Poulouse, A.J. and Kolattukudy, P.E. (1980) Arch. Biochem. Biophys., 201, 313-321.

140. Sheikh, S.S, Mukunda, K. and Katiyar, S.S. (1993) Biochem. Biophys. Acta, 1203, 276-281.

141. Brocklehurst, K. (1982) Methods Enzymol., 87, 427-469.

142. Rakitzis, E.T. (1984) Biochem. J., 217, 341-351.

143. Tian, W.X., Hsu, R.Y. and Wang, Y.S. (1985) J. Biol. Chem., 260, 11375-11387.

144. Simons, S.S., (Jr.), Thompson, E.B., and Johnson, D.B. (1979) Biochemistry, 18, 4915-4922.

145. Blaner, W.S. and Churchich, J.E. (1979) J. Biol. Chem., 24, 1794-1798.

146. Maratihalli, G.B. and Bhagwat, A.S. (1990) Ind. J. Biochem. Biophys., 27, 141-145.

147. Roustan, C., Brevet, A. and Pradel, L.-A. (1973) Eur. J. Biochem., 39, 371-379.

148. Somerville, L.L. and Quiocho, F.A. (1977) Biochim. Biophys. Acta, 481, 493-499.

149. Bayley, H. and Knowles, J.R. (1977) Methods Enzymol., 46, 69-114.

150. Sheikh, S. and Katiyar, S.S. (1993) J. Enzyme Inhibition, 8, 39-50.

151. Rider, M.H. and Hue, L. (1989) Biochem. J., 262, 97-102.

152. Brand, L. and Witholt, B. (1967) Methods Enzymol., 11, 776-856.

153. Schafer, H.-J. and Dose, K. (1984) J. Biol. Chem., 259, 15301-15306.

154. Roth, M. (1971) Anal. Chem., 43, 880-882.

155. Benson, J.R. and Hare, P.E. (1975) Proc. Natl. Acad. Sci. USA, 72, 619-622.

156. Nan'ya, S., Tange, T. and Maekawa, E. (1985) J. Heterocyclic Chem., 22, 449-451.

157. DoMinh, T., Johnson, A.L., Jones, J.E. and Senise, P.P. (Jr.), (1976) J. Org. Chem. 22, 449-451.

158. Palaczewski, K., Hargrave, P.A. and Kochman, M. (1983) Eur. J. Biochem., 139, 429-435.

159. Simons, S.S. (Jr.) and Johnson, D.F. (1978) J. Org. Chem., 43, 2886-2891.

160. Chen, C.-Y., Emig, F.A., Scharmm, V.L., and Ash, D.E. (1991) J. Biol. Chem., 266, 16645-16652.

161. Kobayashi, M., Miura, M., Watanabe, T. and Ischishma, E. (1991) Arch. Biochem. Biophys., 289, 350-354.

162. Puri, R.N., Bhatnagar, D.B. and Roskoski, R. (Jr.), (1985) Biochemistry, 24, 6499-6508.

163. Puri, R.N., Bhatnagar, D.B. and Roskoski, R. (Jr.), (1985) Biochemistry, 24, 6508-6514.

CHAPTER II

MAINTENANCE OF CULTURE, OPTIMIZATION OF CONDITIONS AND THE EFFECT OF NUTRIENTS ON DEXTRANSUCRASE PRODUCTION

II.1 INTRODUCTION

Leuconostoc mesenteroides NRRL B-512F belongs to family *Lactobacillaceae* and is known to synthesize the enzyme dextransucrase when grown in sucrose rich medium. Dextransucrase belongs to the group of enzymes called glucosyltransferases that catalyzes the formation of dextran from sucrose.

$$n\ \text{SUCROSE} \xrightarrow{\text{dextransucrase}} (\text{GLUCOSYL})_n + n\ \text{FRUCTOSE}$$

Dextransucrase is an inducible enzyme and its substrate sucrose, the only known inducer of enzyme synthesis [1], is used by the bacterium as a carbon source for growth and also as a substrate for the synthesis of dextran as soon as the dextransucrase is produced in the culture broth.

Various media have been reported for the maintenance of *Leuconostoc mesenteroides* [2-13]. The organism can be maintained and grown in sucrose rich media [2,4,5,12], which are also used for its isolation. Jeanes [9,10] described two different media for the maintenance of stock cultures. *Lactobacillus* MRS medium is traditionally used for maintaining the organisms belonging to family *Lactobacillaceae* [8]. This medium is also used for long term preservation of *Leuconostoc mesenteroides*. El-Sayed *et al.* [13] described a medium (Tomato-tryptone medium) for culture maintenance. For a short period, the organism can also be stored in enzyme production medium as described by Tsuchiya *et al.* [14]. Although, there are many reports suggesting different media but there is no systematic study on the effect of maintenance media on the enzyme production by *Leuconostoc mesenteroides* NRRL B-512F. In the present study, an attempt has been made to ascertain the extent to which the yield of enzyme is regulated by the media used for maintaining the cultures of *Leuconostoc mesenteroides* NRRL B-512F.

A series of reports are available on the production of dextransucrase under different culture conditions [1,5-7,9,11, 13-23]. The temperatures in the range 20 to 30°C have been used for dextransucrase production by various researchers [1,5-7,9, 11,13-23]. In the present study the temperature influence on the dextransucrase production was studied in a close range. There have been conflicting reports on the production of

dextransucrase using shaken and static flask cultures [5, 7, 9,10,14,15]. It was demonstrated that the aeration and the agitation of culture do not favor the production of dextran as well as the enzyme dextransucrase [6,20-22], However, mild aeration and agitation have been used with uncontrolled pH in batch cultures [5,7,9-11,14] and with controlled pH in the fed-batch [20,22,23], semi-continuous [19] and continuous processes [11,21]. This led us to re-investigate and compare the enzyme production in still and shaken cultures.

Different combinations of temperature, pH and sucrose concentration have been reported for maximum activity of dextransucrase [5,7,9-11,13,14,15,18-26]. In the present study, certain optimal conditions for the assay of dextransucrase activity also have been re-defined.

A large number of reports are available on the production of dextransucrase under different culture conditions using different enzyme production media [5-7,9,11,13-23]. For dextran production by whole cultures, generally high sucrose concentrations have been used [2-5]. Hehre [5] used a medium containing 4% sucrose, sodium chloride, phosphate, and bacto-peptone for dextran production. Tsuchiya *et al.* [14] studied the effects of concentration of sucrose, corn steep liquor and phosphate on the dextransucrase production. They optimized the composition of ingredients and recommended an optimum concentration of 2% of each sucrose, corn steep liquor

and phosphate for dextransucrase production. A combination of certain inorganic salts as micronutrients was also used [15]. El-Sayed *et al.* [13] used a medium similar to that described by Tsuchiya *et al.* [14]. Although, number of reports are available on the effect of various salts on the dextransucrase release from *Streptococcus sobrinus* [27] and *Streptococcus mutans* [28,29], not much information is available on the effects of nutrients on the production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. In the present study the effects of some nutrients on the enzyme production were studied.

II.2 MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides NRRL B-512F was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. Ingredients of the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. For reducing sugar estimation, 3,5-Dinitrosalicylic acid (DNS) and Folin's reagent for protein estimation, were from Sigma Chemical Company, (St. Louis, USA). All other chemicals used were of highest purity grade, commercially available.

Methods

II.2.1 Analytical procedures

II.2.1.1 *Preparation of DNS reagent for reducing sugar estimation*

3,5-Dinitrosalicylic acid (DNS) reagent was prepared as per the method described by Sumner and Sisler [30]. Sodium potassium tartarate (Rochelle salt, 183 g) was dissolved in 500 ml of hot water and mixed with 262 ml of 2 N NaOH. DNS (6.3 g) was slowly added to above mixture and stirred continuously till DNS was dissolved. Redistilled phenol (5 g) and anhydrous sodium metabisulphite (5 g) were added to the above reagent mixture which and cooled to room temperature. The volume was made upto 1 l and stored in an amber color bottle.

II.2.1.2 *Reducing sugar estimation*

To 1.0 ml sample, containing reducing sugar (0.2-0.8 mg/ml), 3.0 ml of DNS reagent with 1.5 ml distilled water were added. This was heated for 5 min on a water bath and cooled to room temperature. The volume was then made up to 15 ml and the optical density (OD) was read at 540 nm using a UV/visible spectrophotometer (Gilford, Model-260, USA) against a blank.

II.2.1.3 *Estimation of sucrose*

To 0.5 ml of samples 1 ml of 1 N HCl was added and heated in boiling water bath for 10 min. After cooling to room temperature 1 ml of 1 N NaOH and 3 ml of DNS reagent were added. This was heated again in boiling water bath for 5 min, cooled to room temperature in tap water and then 9.5 ml of water was added. The absorbance was measured at 540 nm on spectrophotometer against a blank. The standard was plotted using D-fructose.

II.2.1.4 *Preparation of reagents for protein estimation*

The protein content was determined by Lowry method [31] using bovine serum albumin (BSA) as the standard.

Reagents for Lowry method :

Reagent A	:	sodium hydroxide (0.4 g) and sodium bicarbonate (2.0 g) were dissolved in water and the volume made up to 100 ml.
Reagent B1	:	2 % sodium potassium tartrate
Reagent B2	:	1% copper sulfate
Reagent C	:	Prepared fresh by mixing 1.0 ml of reagent B1 and 100 ml of reagent A followed by addition of 1.0 ml of reagent B2.
Phenol reagent	:	1 N phenol reagent

II.2.1.5 *Estimation of protein*

To 1.0 ml sample containing protein or BSA, 5 ml of reagent C were added. After 10 min, 0.5 ml of phenol reagent was added and mixed and the optical density (OD) was measured after 30 min at 660 nm against a blank.

The concentration of protein was calculated as follows:

$$\text{Concentration of protein (mg/ml)} = \frac{\Delta(A_{660}) \times C}{V} \quad \text{(mg/ml)}$$

Where;

C = 1 OD equivalent of BSA from standard plot

$\Delta(A_{660})$ = change in absorbance of the sample.

V = volume of the sample (ml)

II.2.2 Assay of dextransucrase activity

The dextransucrase assay was performed at 30°C in 0.2 M sodium acetate buffer (pH 5.2). The enzyme activity was determined by measuring the production rate of reducing sugar. The assay mixture (3.0 ml) contained 10% substrate sucrose in 0.2 M acetate buffer (pH 5.2) and the enzyme solution. The reaction mixture was incubated at 30°C for 1 h. Aliquots (0.1-0.2 ml), from the reaction mixture were analyzed for reducing sugar as described in Section II.2.1.

II.2.3 Calculation of dextransucrase activity

The activity of enzyme is expressed as Units/ml and the specific activity as Units/mg of protein. One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 μmole of reducing sugar per minute.

The dextransucrase activity was calculated as follows:

$$\text{Enzyme activity (Units/ml)} = \frac{\Delta(A_{540}) \times C \times V}{180 \times t \times v} \quad (\mu \text{ mole/min/ml})$$

Where;

Δ (A_{540}) = change in absorbance of the sample at 540 nm

C = 1 OD equivalent fructose from standard plot

V = volume of reaction mixture (ml)

t = time of reaction (min)

180 = molecular weight of fructose

v = volume of reaction mixture for DNS (ml)

II.2.4 Cell-associated dextransucrase activity assay

The samples (5 ml) from fermenting broth were withdrawn and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used for extracellular enzyme activity as described earlier. The pellet containing the cells was washed with acetate buffer and suspended in 2.5 ml of 0.2 M sodium acetate buffer (pH 5.2). To this, 0.5 ml of 60% sucrose was added and incubated at 30°C for 1 h. The amount of reducing sugar released was determined as described in Section II.2.1.

II.2.5 Growth measurement

Cell growth was determined by diluting broth samples 10 times with 0.9% (w/v) sodium chloride and measuring the turbidity in terms of optical density (OD) at 590 nm against a sterile blank of the initial fermentation medium. The optical density was measured using UV-Vis spectrophotometer Gilford 260 (USA). Cell dry weight, was determined by filtering broth samples through dried and preweighed 0.2 μm filters and drying to constant weight, comparing with blanks of sterile media. The results were plotted against the optical density.

II.2.6 Sterilization and aseptic techniques

All the culture media were sterilized by autoclaving at a steam pressure of 15 lb/in^2, corresponding to a temperature of 121°C for 20 min. All inoculum preparation and culture transfers were carried out under totally aseptic conditions in a laminar air flow cabinet.

II.2.7 Maintenance media

Six different media (Table II.1) were used for maintaining and weekly subculturing *Leuconostoc mesenteroides* NRRL B-512F. The organism was transferred four times in the indicated medium prior to transfer into the enzyme production medium.

Table II.1

Composition (g/l) of different media used for maintaining *Leuconostoc mesenteroides* NRRL B-512F

	Media[a]					
	A	B	C	D	E	F
Sucrose	140	100	20	20	-	20
Glucose	10[†]	-	-	-	20	-
Yeast extract	-	-	5	20	5	5
Beef extract	-	0.5	-	-	10	10
Peptone	10	0.01	-	-	10	10
Tryptone	-	-	2.5	-	-	-
K_2HPO_4	-	1	20	20	2	2
Sodiun acetate	-	-	-	-	5	5
Triammonium citrate	-	-	-	-	2	2
Tween 80	-	-	-	-	1	1
NH_4NaHPO_4	-	3	-	-	-	-
$MgSO_4.7H_2O$	-	0.1	-	0.2	0.2	0.2
$MnSO_4.4H_2O$	-	-	-	0.01	0.2	0.2
$FeSO_4.7H_2O$	-	0.01	-	0.01	-	-
$CaCl_2.2H_2O$	-	-	-	0.01	-	-
p-Aminobenzoic acid	-	0.05	-	-	-	-
KCl	-	0.1	-	-	-	-
NaCl	1	-	-	0.01	-	-
Sugarcane juice[b]	11	-	-	-	-	-
Agar	20	20	20	15	15	15
pH	7.0	7.6	7.4	6.9	6.4	6.4

Footnotes of Table II.1 on next page

[a]A, Bhatnagar *et al.* [12]; B, Stacey [2]; C, Jeanes [9]; D, Tsuchiya *et al.* [14]; E, DeMan *et al.* [8]; F, Modified *Lactobacillus* MRS

[b]Fresh sugarcane juice was filtered and centrifuged at 4000 rpm for 15 min to remove bagasse and other suspended impurities. The pH was adjusted to 7.0 by 10 N KOH. It was then boiled for 5 min, cooled and centrifuged to remove precipitated proteins and pH was again brought to 7.0. The clear supernatant was diluted with distilled water to 15 per cent total solids (Brix 15), checked for neutrality and sterilized.

[†]Invert sugar

II.2.8 Enzyme production medium

The composition of enzyme production medium is shown in Table II.2. The medium was sterilized by autoclaving at a steam pressure of 15 lb/in^2 for 20 min.

Table II.2

Composition (g/l) of enzyme production medium

Sucrose	20
Yeast extract	20
K_2HPO_4	20
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.01
$FeSO_4.7H_2O$	0.01
$CaCl_2.2H_2O$	0.01
NaCl	0.01
pH	6.9

II.2.9 Production of enzyme

The enzyme was produced by the method of Tsuchiya *et al.* [14] and the composition of medium is shown in Table II.2 For inoculum build up a loopful of culture from stab or slant was transferred to 5 ml of enzyme production medium (EPM) and incubated at 23°C. The ratio of inoculum to medium was kept at 1:20. Unless stated otherwise, all fermentations were carried out in triplicate sets of 100 ml EPM in 250 ml Erlenmeyer flask incubated at 23°C in static condition. The samples (5 ml) were withdrawn at appropriate time intervals and centrifuged at 10,000 rpm for 10 min at 4°C to separate the cells. The supernatant was analyzed for enzyme activity as described in Section II.2.2.

II.2.10 Production of dextransucrase under different culture conditions

II.2.10.1 *Effect of temperature*

Leuconostoc mesenteroides NRRL B-512F was grown in static flask batch cultures as described in Section II.2.9. The fermentation was carried out in 100 ml EPM in 250 ml flasks at 20°C, 23°C and 25±0.2°C. The aliquots (5.0 ml) from broth were drawn at appropriate time intervals and centrifuged to remove the cells. The supernatant was analyzed for enzyme activity, as described in Section II.2.2.

II.2.10.2 *Effect of shaking*

The enzyme production was carried out in batch cultures of 100 ml medium in 250 ml flasks. The enzyme production under static condition was compared with the shaken flask at 23±0.2°C. The shaking was done on a rotary shaker at 120 rpm keeping other conditions unchanged. The aliquots (5.0 ml) from broth were withdrawn at indicated time intervals and centrifuged to remove the cells and supernatant analyzed for enzyme activity, as described in Section II.2.2.

II.2.11 Purification of dextransucrase

The crude dextransucrase with an activity of 1.4 U/ml was purified by fractionation with polyethylene glycol 400 as reported earlier in Section III.2.4.

II.2.12 Optimization of conditions for activity assay

II.2.12.1 *Effect of temperature*

The enzyme activity of purified dextransucrase was determined at temperatures varying from 20°C to 37°±0.2°C.

II.2.12.2 *Effect of pH*

The activity of dextransucrase at different pH ranging from 4.6 to 5.6 was determined using 0.2 M acetate buffer.

II.2.12.3 *Effect of sucrose concentration*

The dextransucrase activity was determined by varying the concentration of sucrose from 2-20% to find the optimum concentration of substrate for maximum enzyme activity.

II.2.13. Effect of Nutrients on dextransucrase production

II.2.13.1 *Effect of sucrose*

The effect of sucrose on the enzyme production was studied by varying the concentration of sucrose in the medium from 1% to 5%. The medium described in Table II.1, D containing 2% sucrose was taken as control.

II.2.13.2 *Effect of yeast extract and* K_2HPO_4

The effect of yeast extract was studied in combination with the phosphate. The concentration of yeast extract was varied as 1.5, 2, 3 and 4%, where the control medium contained 2% yeast extract. The phosphate concentration was varied as 1.5, 2 and 2.5% taking 2% yeast extract as the control.

II.2.13.3 *Effect of peptone and beef extract*

The effect of peptone and beef extract on the enzyme production was studied by varying their concentration in the medium from 0.1 to 1% and 0.5 to 2%, respectively. The medium described in Table II.1, D with out peptone and beef extract was used as a control medium.

II.2.13.4 *Effect of tween 80*

The effect of tween 80 on enzyme production was studied by varying its composition in the range 0.1-0.5% taking the control medium with no tween 80.

II.2.13.5 *Effect of salts on enzyme production*

The effect of metal ions on enzyme production was studied by varying their composition in the EPM. The concentration of $MgCl_2$ and NaF were varied in the range 1-100 μM. The medium described in Table II.1, D was used as control.

II.2.13.6 *Effect of pulse feed of sucrose on enzyme production*

The fermentation was carried out in 1 l of EPM (Table II.2) in a 2 l conical flask. All other culture conditions were similar as described in Section II.2.9. After 22 h of incubation when the enzyme activity began to fall, a pulse of (50 ml containing 20 g of sucrose and 20 g of dipotassium hydrogen orthophosphate) was added aseptically. Three such more pulses of sucrose were given after every 4 h.

II.3 RESULTS AND DISCUSSION

II.3.1 Screening of maintenance media

Leuconostoc mesenteroides NRRL B-512F maintained in different media was grown in enzyme production medium (EPM) under identical culture conditions and dextransucrase activity was determined. Culture maintained in modified MRS medium (Table II.1, F) gave maximum activity of 5.2 U/ml (Fig. II.1) whereas, media with higher sucrose content (Table II.1, A&B) could result in the activities of 3.8 U/ml and 4.2 U/ml, respectively. The cultures maintained in high sucrose medium when transferred to EPM gave much lesser cell growth as compared to the cultures maintained in low sucrose medium. The reduced enzyme activity in the cultures maintained in sucrose rich media might be the consequence of reduced biological activity of the organism induced by the rapid substrate exhaustion in the maintenance medium. Culture in this state, when transferred to the EPM was not able to produce higher enzyme activity. This makes the sucrose rich media unsuitable for long term maintenance of the organism. Medium C (Table II.1) described by Jeanes [9] did not lead to higher enzyme production which may be because of the absence of micro nutrients. The organism could be conveniently maintained in low sucrose media (Table II.1, D&F) or even glucose containing medium (Table II.1, E) as these cultures when transferred to EPM, produced appreciably higher enzyme activity

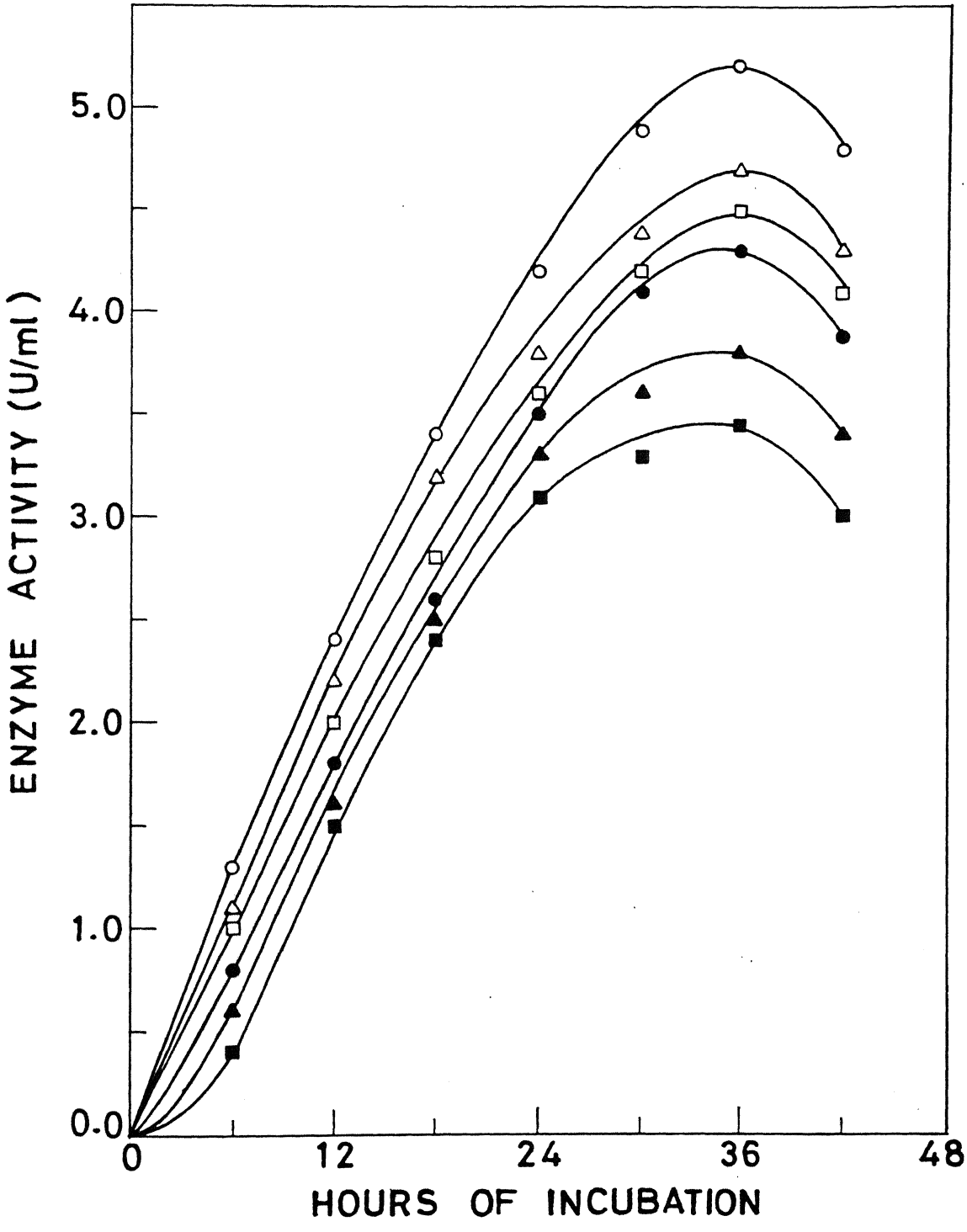


Figure II.1. Time dependent production of exocellular dextransucrase in enzyme production medium (Table-II.1, D). Exocellular enzyme activity profile with time when *Leuconostoc mesenteroides* NRRL B-512F was maintained in media (refer Table II.1). F (O), E (Δ), D (□), C (●), B (▲), A (■).

(Fig. II.1). Maintaining the trend, modified MRS medium (Table II.1, F) gave maximum dextransucrase activity of 5.2 U/ml in the culture supernatant. These results showed that media containing low sucrose induced higher enzyme activity than the media containing high sucrose content.

In a separate experiment, enzyme activity was monitored, when the organism maintained in modified MRS medium (Table II.1, F), a sucrose rich medium (Table II.1, B) and in an intermediate transfer from F to B medium were grown in EPM. The extracellular enzyme activity decreased by 22% and the specific activity by 30% when the organism was transferred from maintenance medium F to B (Table II.3). These observations supported the contention that *Leuconostoc mesenteroides* NRRL B-512F exhibits the capacity for producing enzyme with higher activity when maintained in modified MRS medium and that the presence of higher amount of sucrose in the maintenance medium adversely affects the enzyme production.

It was repeatedly found that the transfer of the culture from modified MRS medium to other media produces the enzyme with lower activity and reverse transfers to the modified MRS medium resulted in an increase in the enzyme activity. The modified MRS medium was found better for prolonged maintenance of the culture and gave higher dextransucrase yield as compared to MRS medium.

Table II.3

Effect of intermediate transfer of *Leuconostoc mesenteroides* NRRL B-512F from low sucrose to high sucrose medium on the activity of extracellular dextransucrase.

Maintenance Medium[a]	Enzyme activity (U/ml)	Specific activity (U/mg)
B	3.9	0.60
F	5.8	1.20
F⟶B	4.5	0.85

[a]Refer Table II.1

There are very few reports on the cell-associated dextransucrase (CADS) activity of *Leuconostoc mesenteroides* NRRL B-512F. It has been reported that the enzyme occurs in the extracellular as well as cell-associated/intra-cellular forms in *Leuconostoc mesenteroides* NRRL B-1299 [33] and *Streptococcus mutans* [34-36]. It was reported that the distribution of dextransucrase in *Streptococcus mutans* and *Streptococcus sobrinus* was dependent on the growth medium [37]. In the present study the effect of maintenance media on the CADS activity was investigated. Fig. II.2 shows the time dependent changes of CADS activity during enzyme production. Some CADS

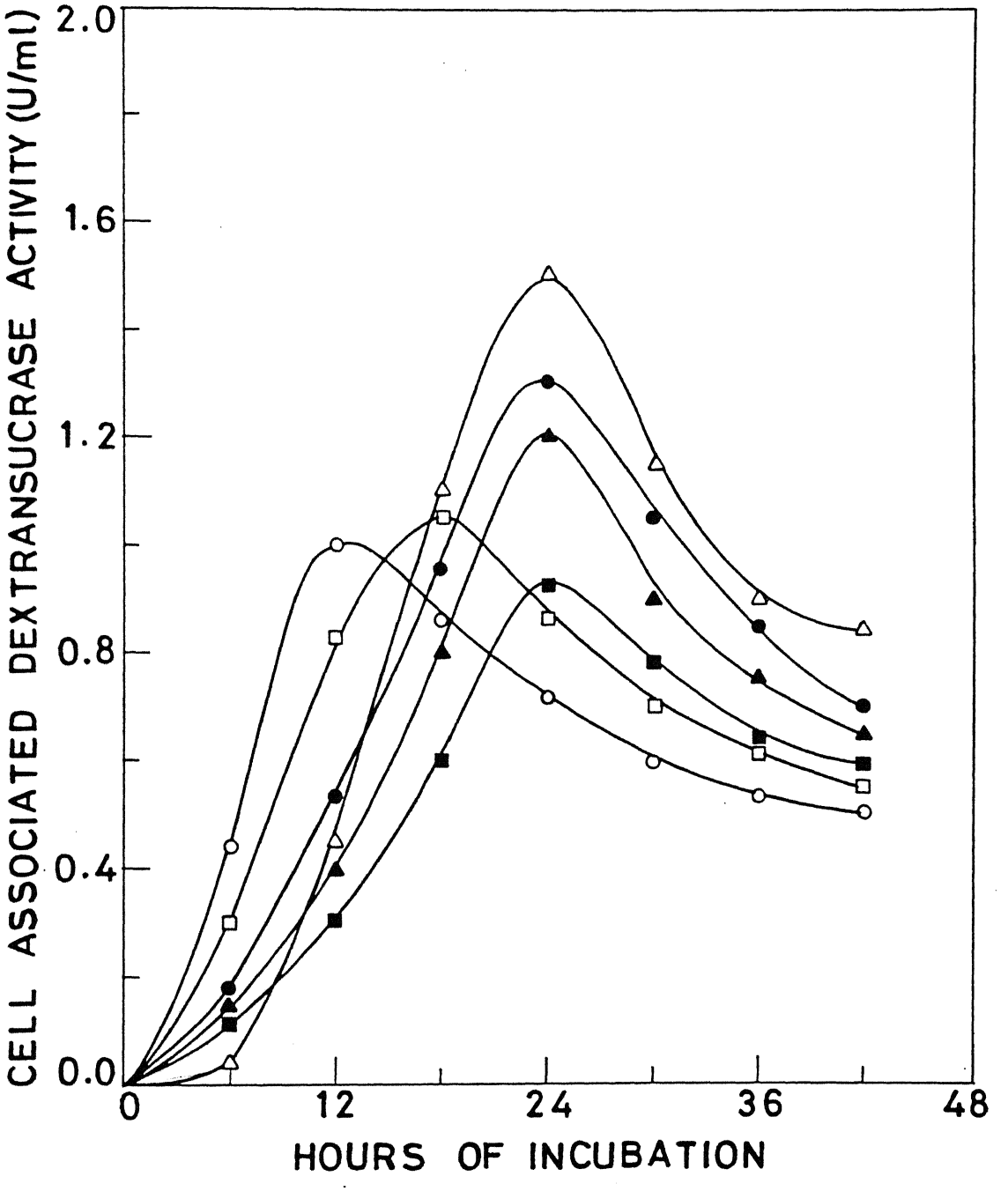


Figure II.2. Time dependent change of cell-associated dextransucrase (CADS) activity. CADS activity profile with time when *Leuconostoc mesenteroides* NRRL B-512F was maintained in media (refer Table II.1). F (O), E (Δ), D (□), C (●), B (▲), A (■).

activity was detected in all cases, however, the ratio of extracellular/cell-associated dextransucrase activity was maximum (9.8) when the organism maintained in modified MRS medium was grown in EPM (Table II.4). Profiles of CADS activity were found to be quite different than those of extracellular enzyme activity. In all the maintenance media, maxima of CADS activity occurred at an early stage as compared to the maxima of extracellular enzyme activity. CADS activity with MRS medium (Table II.1, E) was maximum 1.5 U/ml whereas, with modified MRS medium (Table II.1, F) it was 1.0 U/ml (Fig. II.2). In all the systems, extracellular enzyme activity reached the maximum value when CADS activity had attained lower value. Appearance of maxima of the CADS activity at the earlier stages of fermentation is expected as dextransucrase is a secretory enzyme which is synthesized inside the cell and released in to the medium.

Table II.4

Localization of extracellular dextransucrase (ECDS) and cell-associated dextransucrase (CADS) activity of *Leuconostoc mesenteroides* NRRL B-512F grown in different maintenance media. The cultures were grown in enzyme production medium at 23°C for 36h.

Medium[a]	ECDS activity (U/ml)	CADS activity (U/ml)	ECDS/CADS
A	3.4	0.63	5.7
B	3.6	0.70	5.4
C	4.0	0.75	5.3
D	4.5	0.60	7.5
E	4.6	0.90	5.1
F	5.2	0.53	9.8

[a]Refer Table II.1

II.3.2 Optimization of conditions for enzyme production

II.3.2.1 *Effect of temperature*

The dextransucrase production to a large extent was affected by the temperature of incubation. The optimum temperature of incubation for the enzyme production was found to be 23°C, at which dextransucrase activity was 5.3 U/ml (Fig. II.3A). Table II.5 summarizes the results of the effect of temperature on dextransucrase production. The enzyme activity obtained with fermentation at 23°C was 28% and 17% more than the activity obtained at 25°C and 20°C, respectively.

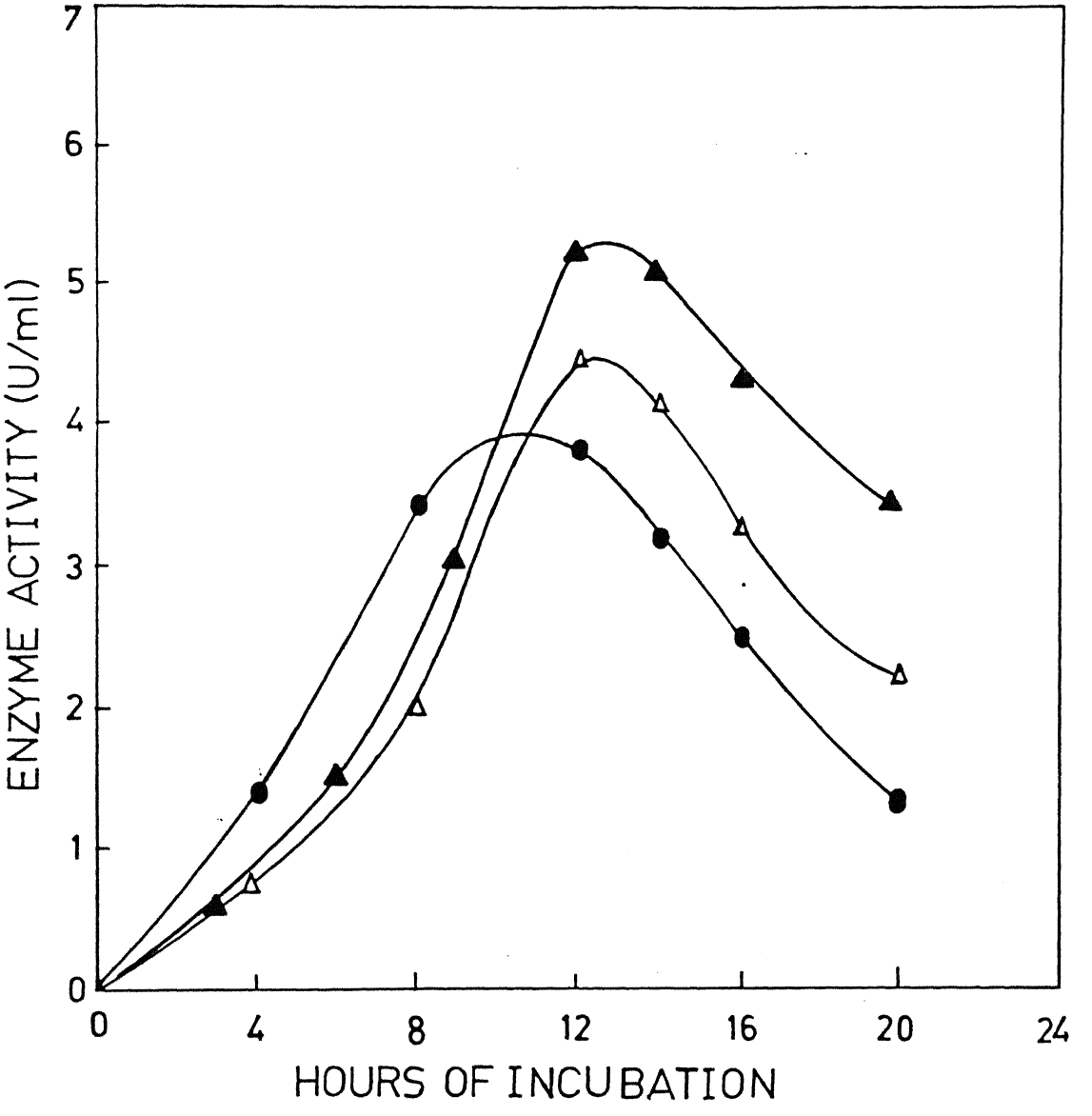


Figure II.3A. Effect of temperature dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F in static flask cultures. Enzyme activity profile with time at temperatures: 20°C (Δ), 23°C (▲), 25°C (●).

The temperature of fermentation is a critical parameter and interdependent with the pH of the broth. Although, the growth rate was higher at 25°C (Fig. II.3B), enzyme yield was lower than that observed at 23°C (Table II.5). The pH of fermenting broth was in the range of 6-6.5, when the maximum enzyme yield was observed (at 12h) at all the three temperatures studied. Earlier reports also suggested that the enzyme production was maximum in the pH range of 6.0-7.0 and that the higher temperatures keeping the pH unchanged resulted in progressive inactivation of enzyme and thus lower yields were obtained [38]. In the present study it was observed that a temperature higher than optimal temperature of 23°C, reduced the enzyme yield. Below the optimum temperature, the growth of the organism was slower (Fig. II.3B), leading to the delayed and reduced synthesis of the enzyme. The decrease in enzyme activity at all the temperatures at the end of fermentation is attributable to the extreme sensitivity of dextransucrase to unfavorable pH conditions. Moreover, once the rate of enzyme production begins to fall (after 12h), the denaturation of the enzyme occurs faster than its generation culminating in overall reduction in the enzyme activity.

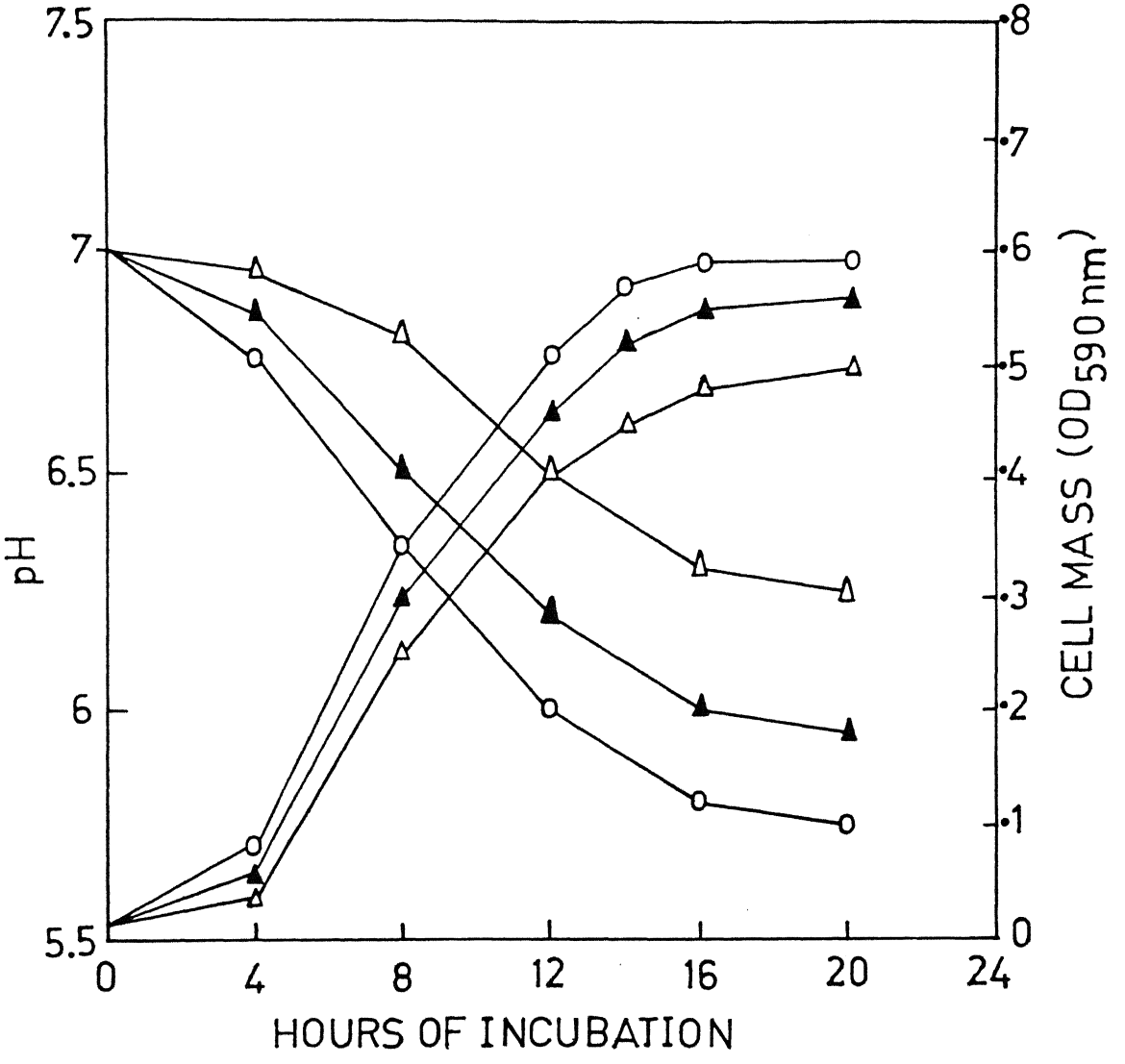


Figure II.3B. Cell growth and pH decrease with time during the dextransucrase production at temperatures: 20°C (Δ), 23°C (▲), 25°C (O).

Table II.5

Effect of temperature and shaken flask culture on dextransucrase production

	Static			*Shaken*
Temperature (°C)	20±0.2	23±0.2	25±0.2	23±0.2
Cell mass (g/l)	9.0	9.7	10.2	10.5
Enzyme activity (U/ml)	4.4	5.3	3.9	3.6
Specific activity (U/mg)	0.77	1.2	0.67	0.80

II.3.2.2 *Effect of shaken culture*

Dextransucrase activity in the static flask culture was 30% higher than that in shaken flask culture (Fig. II.4A). Table II.5 embodies comparative data on enzyme production in the static and rotary shaken flask cultures. In static cultures the enzyme production started later but increased rapidly and resulted in higher enzyme activity. In the static culture, cell growth and fall in pH was slower (Fig. II.4B) which is indicative of some diffusion controlled effect on cell activity. The cells produce dextransucrase and acid, these might be expected to collect in 'pockets' around the cells. This results in lowering of the pH around the cells which is different from that of the bulk medium pH. The lowering of the pH around the cells enhances the enzyme release and also

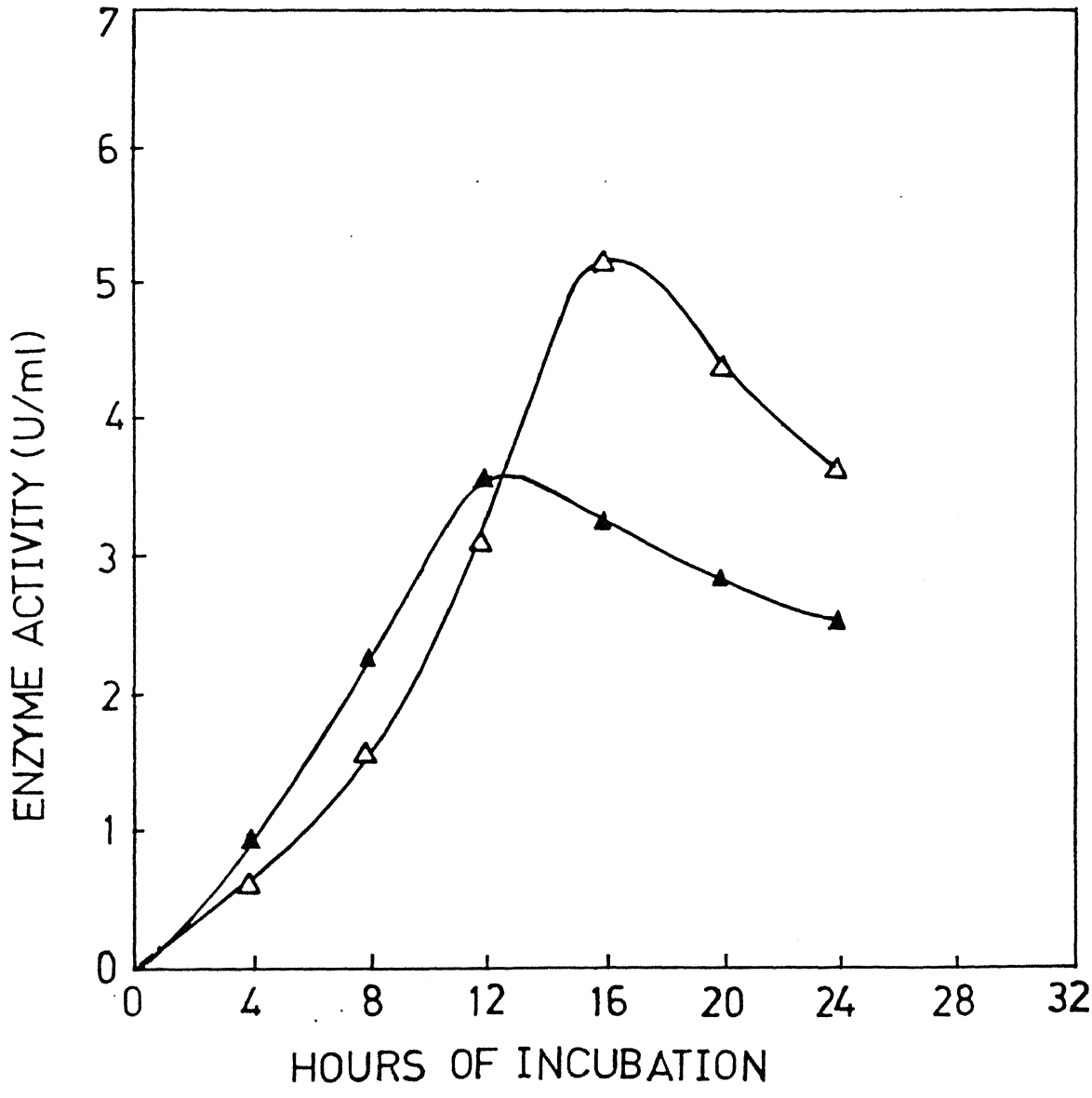


Figure II.4A. Dextransucrase production with time in shaken (▲) and static (Δ) flask cultures at 23°C.

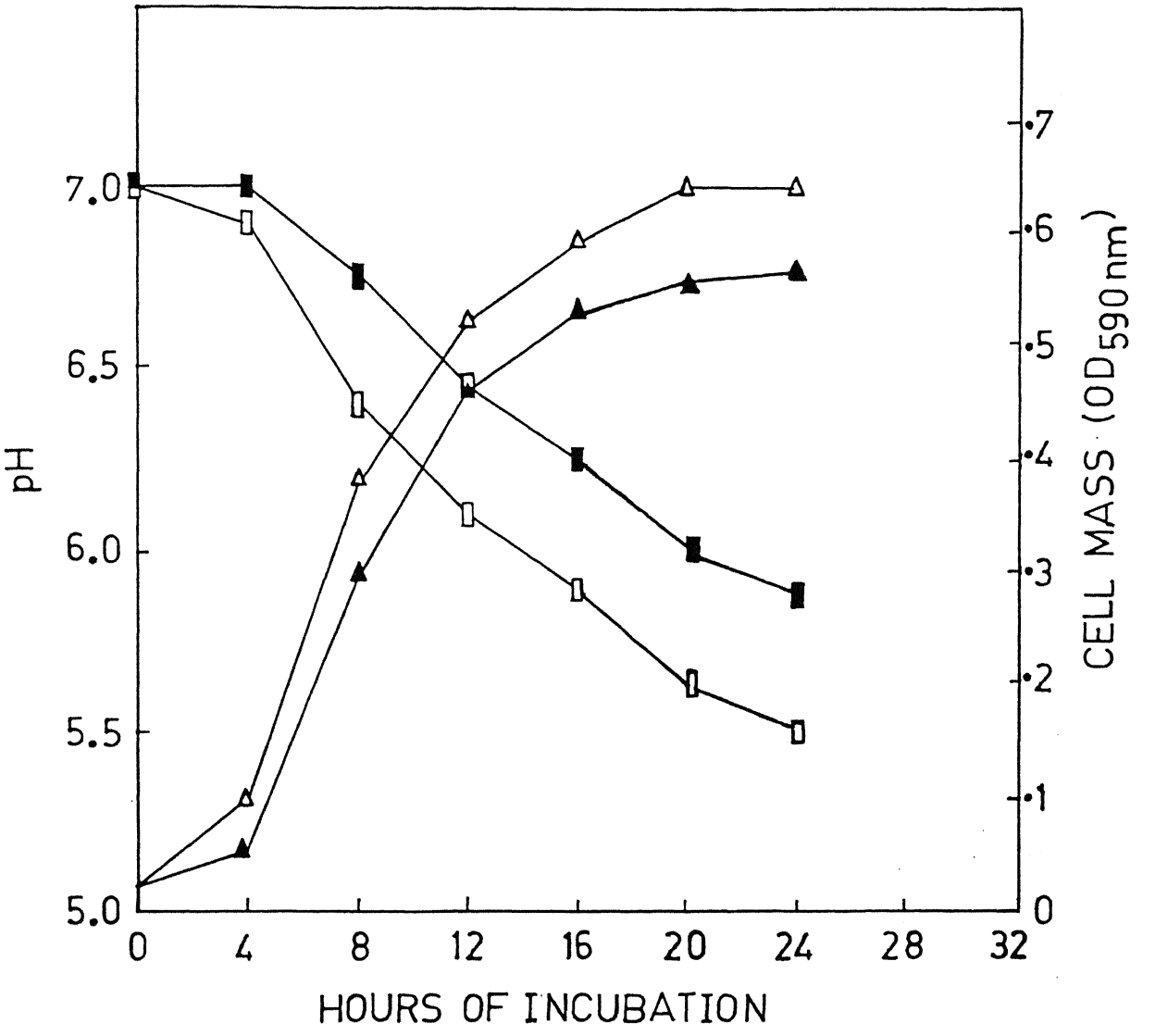


Figure II.4B. Cell growth and pH decrease with time during dextransucrase production in shaken (open symbol) and static (closed symbol) flask cultures at 23°C: Cell growth (▲), (△); pH (■), (□).

stabilizes the enzyme thus resulting in higher activity of enzyme.

In shaken flask cultures the pockets of lower pH around the cells get disturbed, thereby reducing the enzyme activity and yield. Further, it has been demonstrated that the agitation and aeration do not favor the dextransucrase production [20,22]. However, aeration with CO_2 resulted in higher enzyme yield [22]. The CO_2 released by the cells of *Leuconostoc mesenteroides* during fermentation, is present as blanket over the broth, under which the elaboration of dextransucrase is more efficient [22]. Since the bacterium is microaerophilic saprophyte deriving energy through the oxidation of the substrate in the medium, an increase in CO_2 concentration in fermenting medium creates anaerobic zones around the cells requiring oxidation of more quantity of substrate to maintain the required supply of energy. This causes the organism to produce more enzyme. Such a condition is not created in the shaken cultures, thus the production and the activity of enzyme is reduced.

II.3.3 Optimization of conditions for enzyme assay

Dextransucrase is very sensitive to temperature and pH. The purified dextransucrase exhibited maximum activity at 30°C (Fig. II.5). On analysis in a narrow range of pH, the optimum pH was found to be 5.2 (Fig. II.6). The rate of reaction

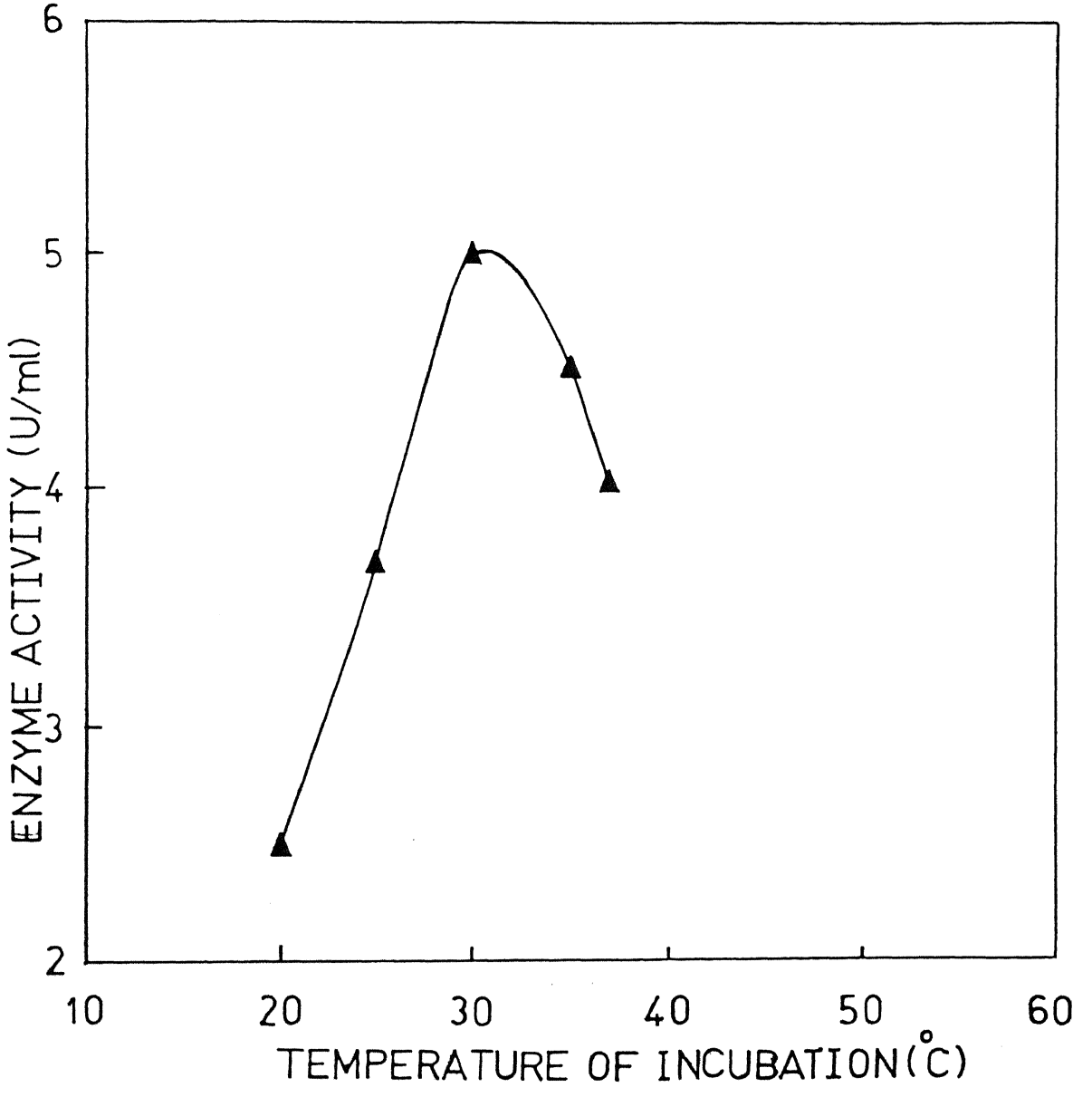


Figure II.5. Effect of temperature on the activity of dextransucrase. The enzyme activity was determined by carrying out the assay in 0.2 M sodium acetate buffer (pH 5.2) and 10% sucrose.

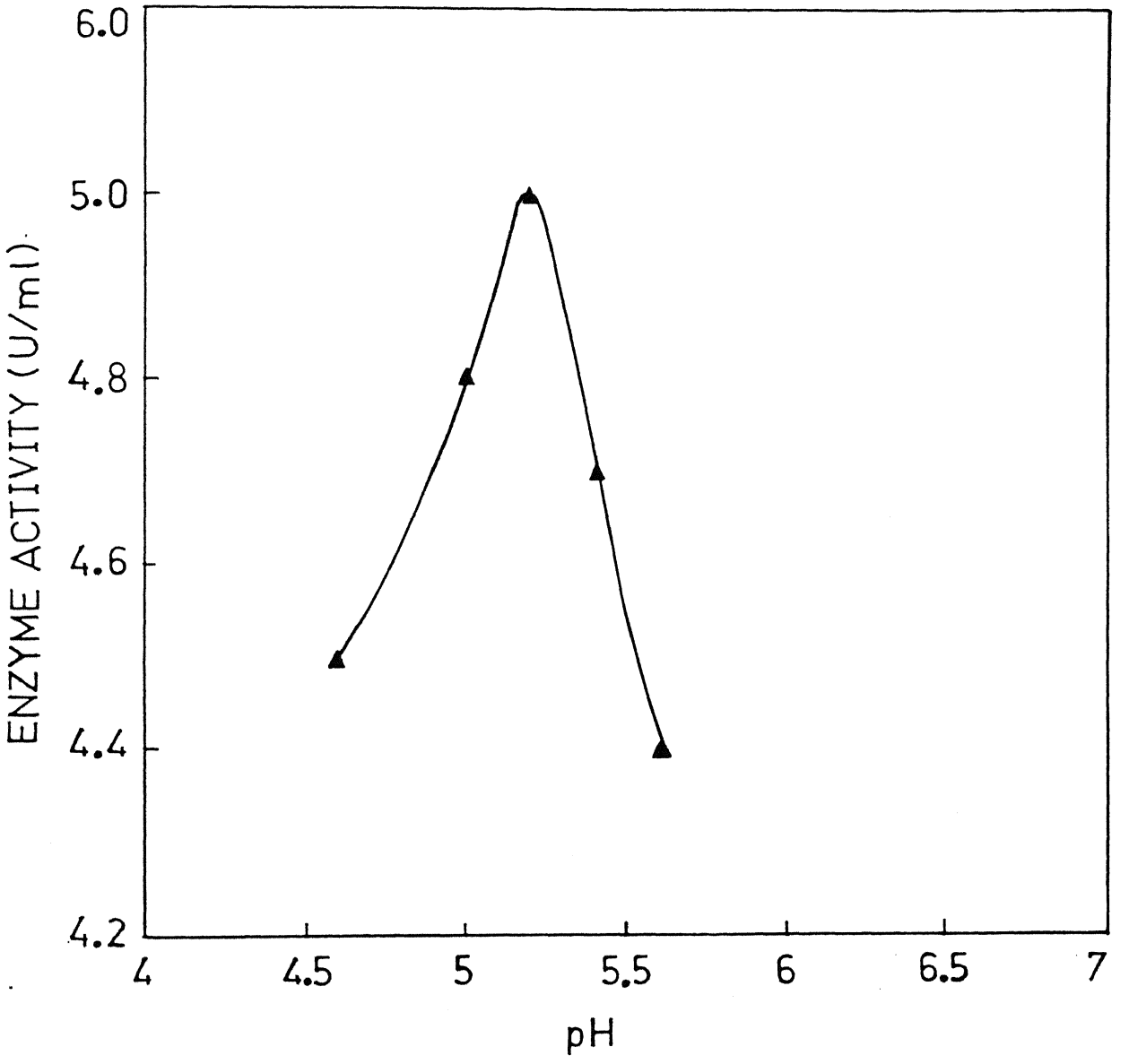


Figure II.6. Activity profile of dextransucrase as a function of pH. The enzyme activity was determined by carrying out the reaction in 0.2 M sodium acetate buffer and 10% sucrose at 30°C.

increased with an increase in the sucrose concentration up to 290 mM but decreased as the sucrose concentration was further increased (Fig. II.7). This trend did not follow completely the Michaelis equation. The K_m value obtained from double reciprocal plot of same data was 47 mM and the V_m was 8.7 U/ml. The results were different from those of Hehre [7], who reported the K_m value of 20 mM, V_m of 0.22 U/ml, optimum pH 5.6 and optimum sucrose concentration of 200 mM for dextransucrase. The result of the effect of sucrose on dextransucrase of the present findings is in accordance with that reported earlier [39]. It has been reported that at a concentration higher than optimum, sucrose inhibits the enzyme, it binds to a third low affinity binding site, allosterically changing the conformation of the active site so that dextran is not synthesized but acceptor products can be synthesized.

II.3.4 Effect of Nutrients

The elaboration of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512F was studied using enzyme production medium, described by Tsuchiya *et al.* [14]. The effects of some nutrients and salts on dextransucrase production were studied. An increase in concentration of sucrose to 4% resulted in the increase of activity of dextransucrase and beyond this concentration the activity decreased (Fig. II.8). With 4% sucrose the activity was 1.7 times more than that observed with

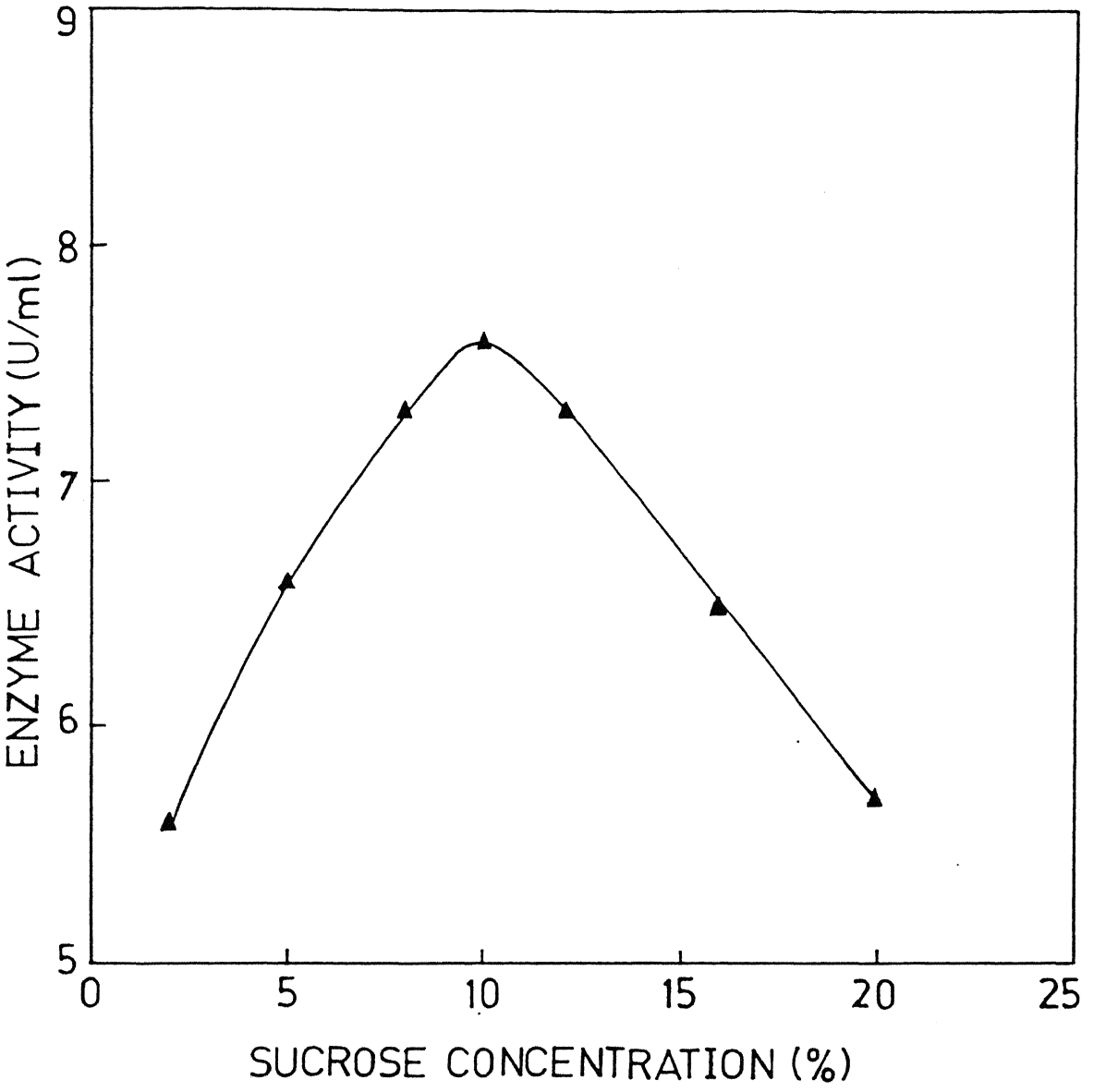


Figure II.7. Effect of sucrose concentration on dextransucrase activity. The enzyme activity was determined in 0.2 M sodium acetate buffer (pH 5.2) at 30°C.

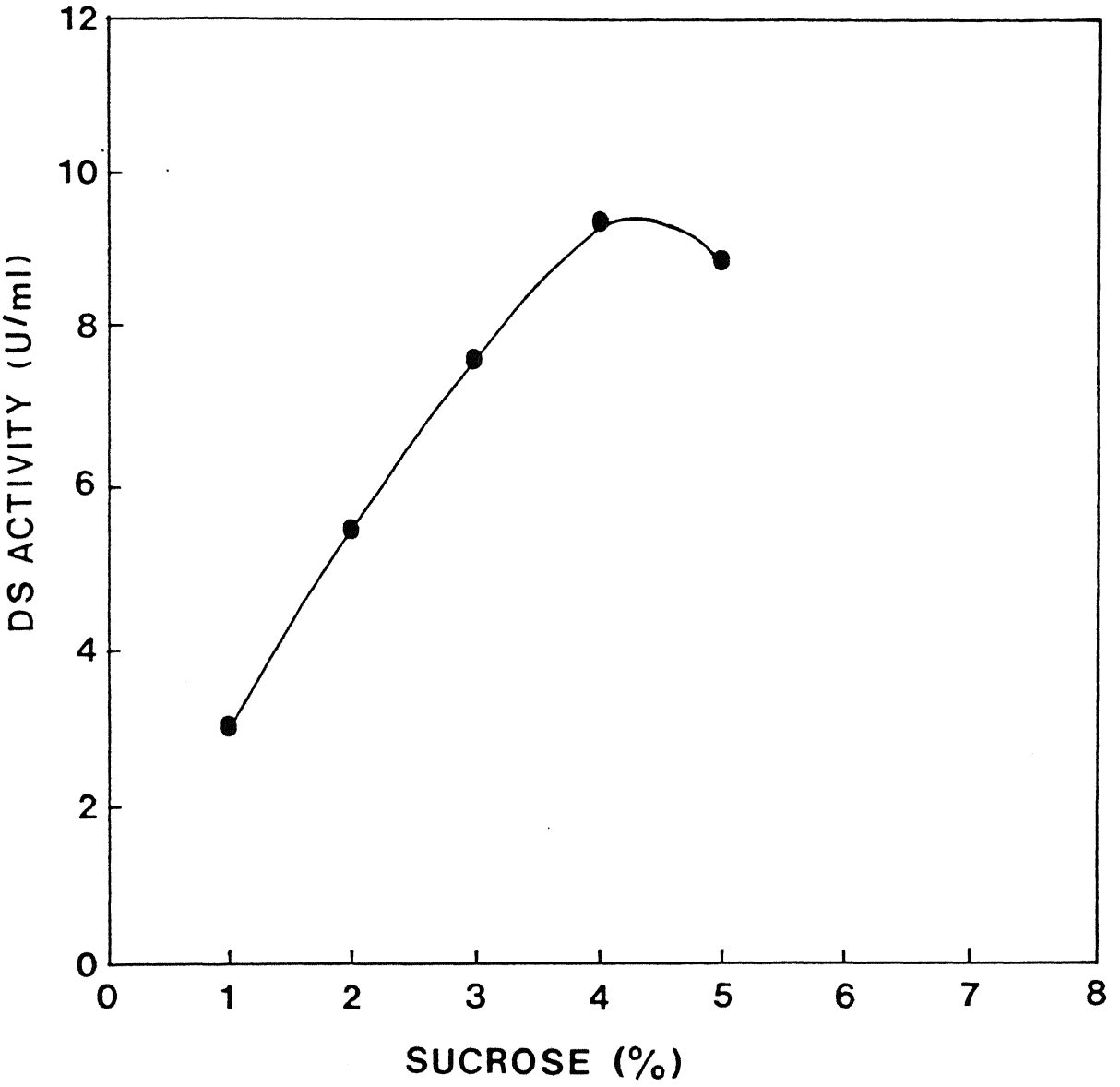


Figure II.8. Effect of sucrose on production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512F.

2% (Table II.6). The broth became more and more viscous with the increase of sucrose concentration due to concomitant formation of dextran. Further, the separation of enzyme from the broth was also difficult. It was inferred that the increase of viscosity of the culture medium interfered with the cell growth and consequently the enzyme production. Similar results on the effect of sucrose on dextransucrase production have been reported earlier [14,40].

The enzyme production was dependent on the concentration of yeast extract and K_2HPO_4. It was observed that at a constant yeast extract concentration, increasing the level of the K_2HPO_4 increased the enzyme activity (Fig. II.9). This effect was more pronounced at lower concentration of yeast extract. With the increase in concentration of K_2HPO_4 from 1.5 to 2.5% the dextransucrase activity enhancement was as high as 1.7 fold when yeast extract level was 1.5% in the medium. A maximum of 6.7 U/ml of dextransucrase activity was obtained with the 1.5% yeast extract and 2.5% phosphate concentration in the medium (Table II.6). Further, enzyme production decreased with an increase in the concentration of yeast extract at a constant phosphate concentration (Fig. II.9). With the increase of yeast extract concentration from 1.5% to 4.0% at a constant 2.5% K_2HPO_4 concentration the dextransucrase activity decreased from 6.7 U/ml to 4.5 U/ml. These observations showed that higher yeast extract concentration does not favor the enzyme

production. It has been reported earlier that enzyme production may be enhanced by increasing the levels of yeast extract as well as K_2HPO_4 [14,20]. It has also been reported that the enzyme production and yield depend on the type of yeast extract used [20]. It was shown that the enzyme yield varied in as much as by 50% using different types of yeast extract. In the present study with the type of yeast extract used, a combination of low yeast extract concentration (1.5%) and higher phosphate levels (2.5%) gave the maximum enzyme production. These results also showed that the enzyme yield depends upon the type of nitrogen source used.

Other nitrogen sources such as tryptone, peptone and barley malt extract have also been used in the medium for dextransucrase production [13,18,41]. However, effect of these nutrients on the enzyme production have not been studied. The effects of peptone and beef extract on dextransucrase production were studied. The presence of peptone (Fig. II.10) and beef extract (Fig. II.11) in the medium in addition to 2% yeast extract resulted in an increased production of dextransucrase. The enzyme activity increased by approximately 30%, by both peptone (1%) and beef extract (2%), as compared to the control (Table II.6). The increase in enzyme production might be due the presence of some additional micronutrients in beef extract and peptone. These results also indicated that dextransucrase production and yield depend upon the type of

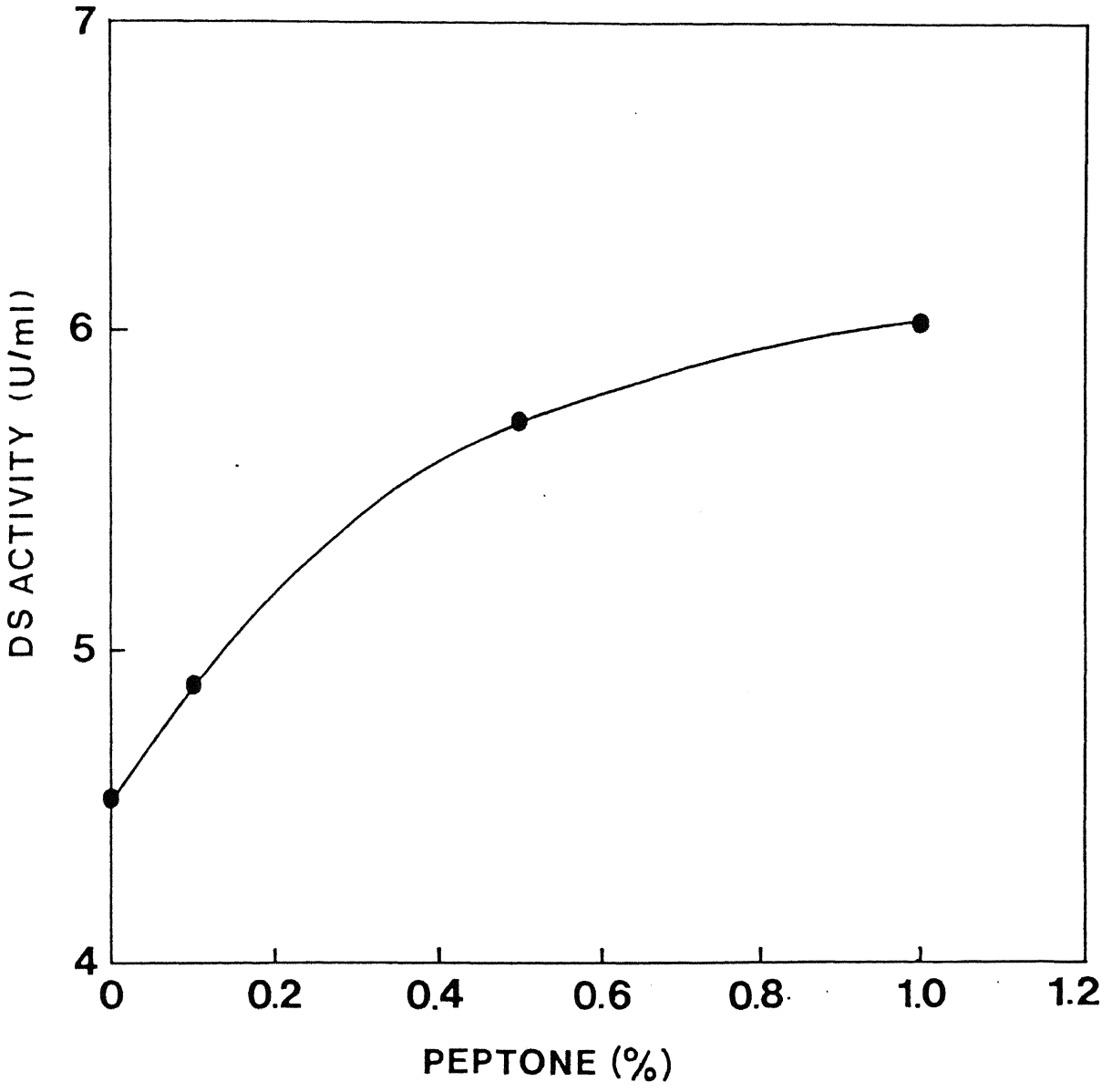


Figure II.10. Effect of peptone on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F.

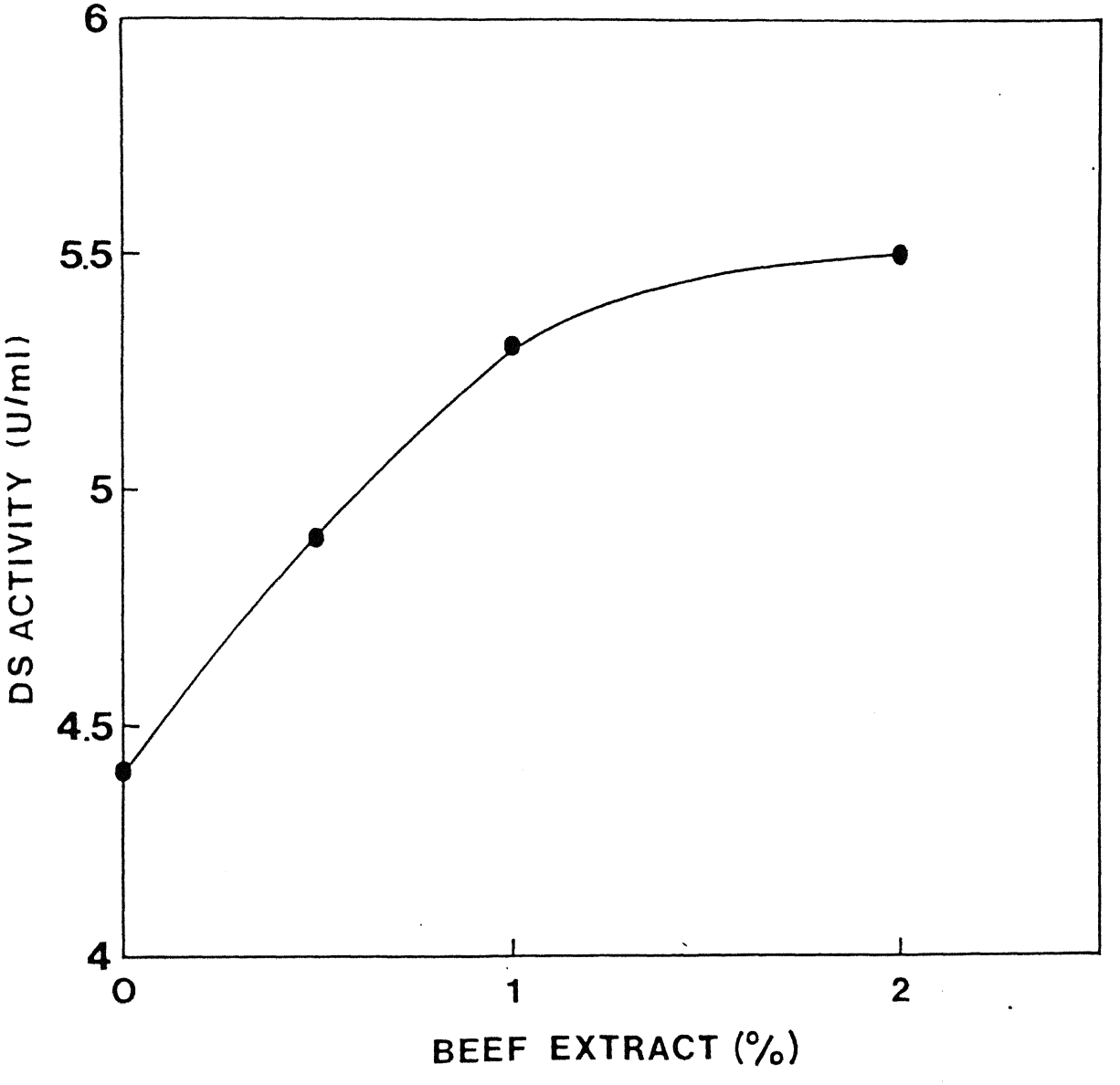


Figure II.11. Effect of beef extract on production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512F.

nitrogen nutrient used.

Table II.6

Maximum activity of dextransucrase achieved with the variation in composition of nutrients. The effects of nutrients were compared with the control medium[a] except in the case of $MgCl_2$ where the control medium contained no $MgSO_4.7H_2O$.

Nutrient (Concentration)	Enzyme activity (U/ml)
Control	100
Sucrose (4%)	175
Yeast extract (1.5%)	115
Peptone (1.0%)	133
Beef extract (2%)	125
K_2HPO_4 (2.5%)	133
Tween 80 (0.5%)	125
$MgCl_2$ (100 μM)	110
NaF (100 μM)	125
Yeast extract (1.5%) + K_2HPO_4 (2.5%)	149

[a]The control medium composition: sucrose, (2%); yeast extract, (2%); K_2HPO_4, (2%); R* Salts (by vol.), (0.5)%;

R* Salts: $MgSO_4.7H_2O$, (4%); $MnSO_4.4H_2O$, (0.2%); $FeSO_4.7H_2O$, (0.2%); $CaCl_2.2H_2O$, (0.2%); NaCl, (0.2%);

Addition of tween 80 to the EPM stimulated the production of enzyme. Although tween 80 had no effect on bacterial growth and *in vitro* dextransucrase activity, the level of dextransucrase in the medium was increased by 25% (Table II.6). Dextransucrase activity increased in medium as the tween 80 concentration increased (Fig. II.12). Accordingly, the increase in the activity reflects an increase in the enzyme level. The enhancement in the activity dextransucrase was significantly less than that obtained with *Streptococcus mutans* [29,42] and *Streptococcus salivarius* [43], where the enhancement of the activity of the dextransucrase giving soluble dextran was 10 to 30 fold in presence of 0.1% tween 80.

The effect of magnesium ions on dextransucrase when present in culture medium at concentrations of 1 to 100 μM is shown in Fig. II.13. Magnesium ions had no effect on cell growth rate as well as the preformed crude enzyme. Dextransucrase activity increased about 1.1 fold in the presence of 100 μM magnesium ions compared to that in the medium with no added magnesium ions (Table II.6). These results are similar to those reported earlier for *Streptococcus sobrinus* [27]. It is known that secretory proteins are synthesized as precursors and that the portion of the NH_2-terminal peptide as a signal peptide is processed during secretion [27]. In *Escherichia coli* two distinct signal peptidases are found. One of them, the leader peptidase, is

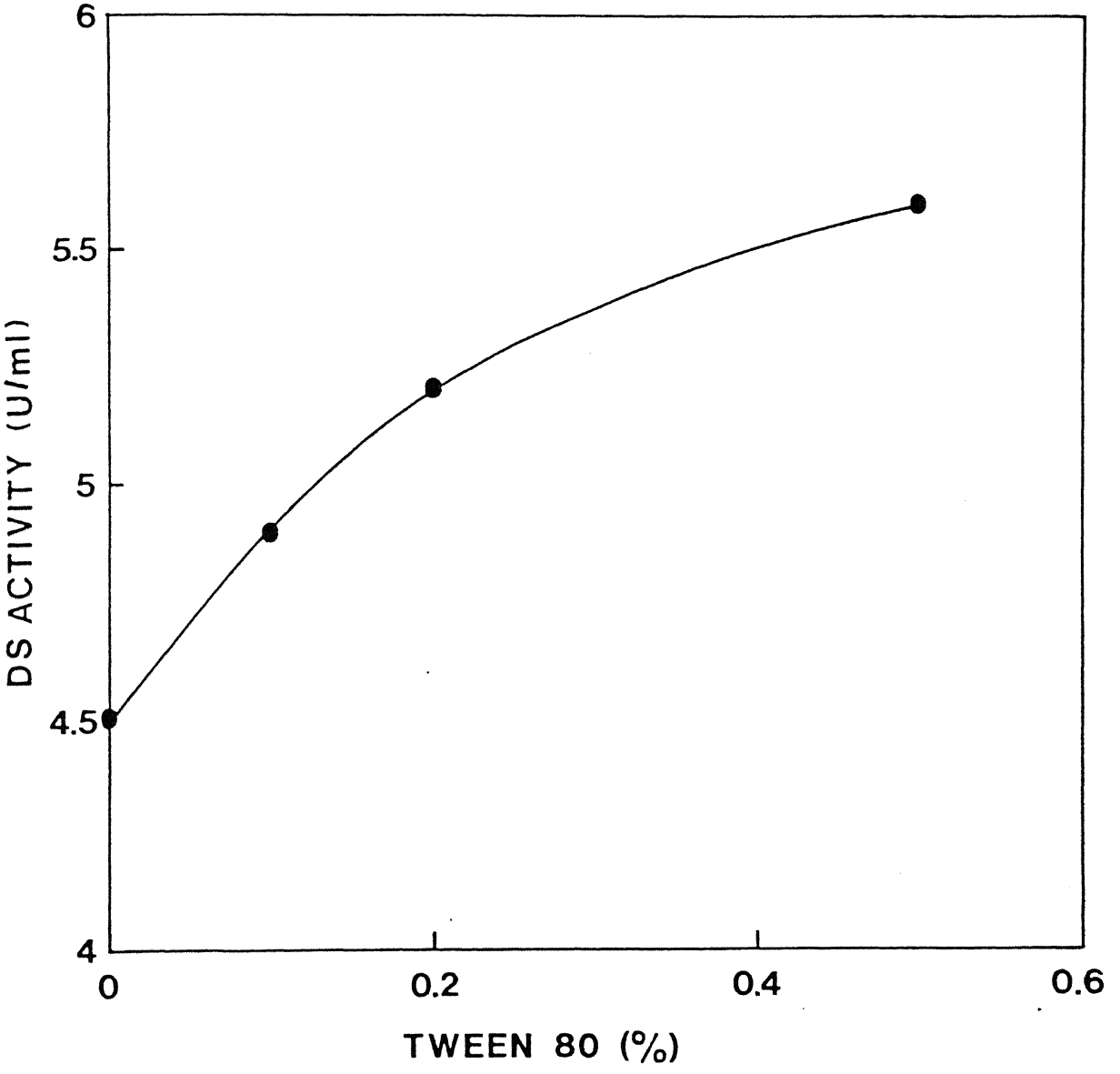


Figure II.12. Effect of tween 80 on production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512F.

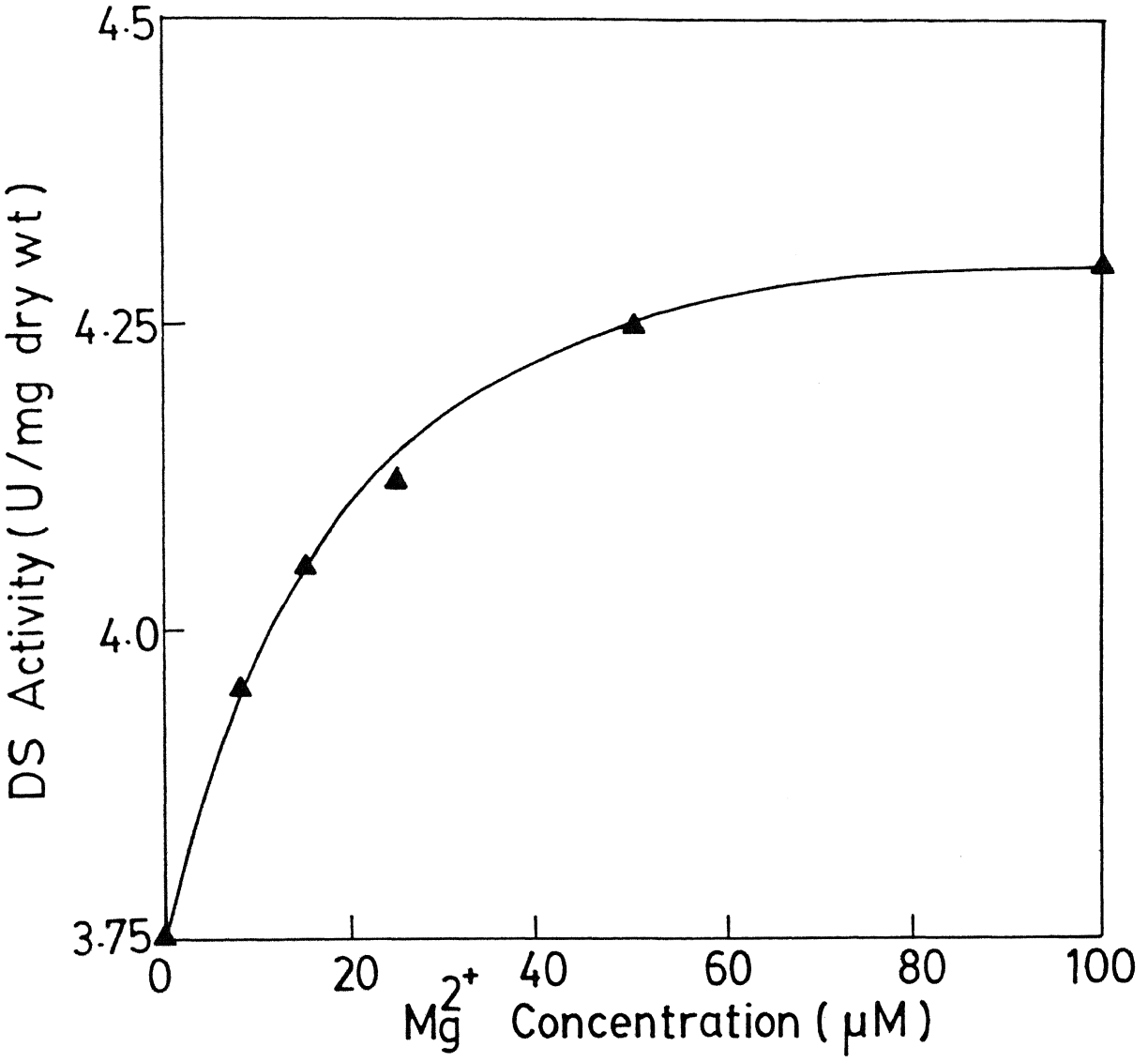


Figure II.13. Effect of $MgCl_2$ present in meidum on extracellular dextransucrase activity from *Leuconostoc mesenteroides* NRRL B-512F.

reported to have broad specificity and is thought to be involved in secretion of various proteins. Magnesium ions are known to inhibit the leader peptidase activity of *Escherichia coli*. The present results of the effect of magnesium ions on dextransucrase secretion indicated that mechanism of enzyme secretion in *Leuconostoc mesenteroides* NRRL B-512F is similar to *Streptococcus sobrinus* and is probably different from that of *Escherichia coli*.

Sodium fluoride (NaF) in the medium enhanced the dextransucrase activity of *Leuconostoc mesenteroides* NRRL B-512F (Fig. II.14). The cell growth rate and the activity of dextransucrase were not affected by NaF. The dextransucrase activity was increased by 25% with 100 μM NaF concentration in the medium as compared to the control medium that contained no NaF (Table II.6). These observations showed that NaF increases the secretion of dextransucrase from cells. Binary effects of NaF have been reported with *Streptococcus mutans*. The extracellular dextransucrase activity leading to the formation of soluble dextran was increased by 10 fold whereas, the enzyme activity leading to the formation of insoluble dextran was decreased. The present findings show that the enhancement of dextransucrase activity by NaF was not as significant as reported for *Streptococcus mutans* [29].

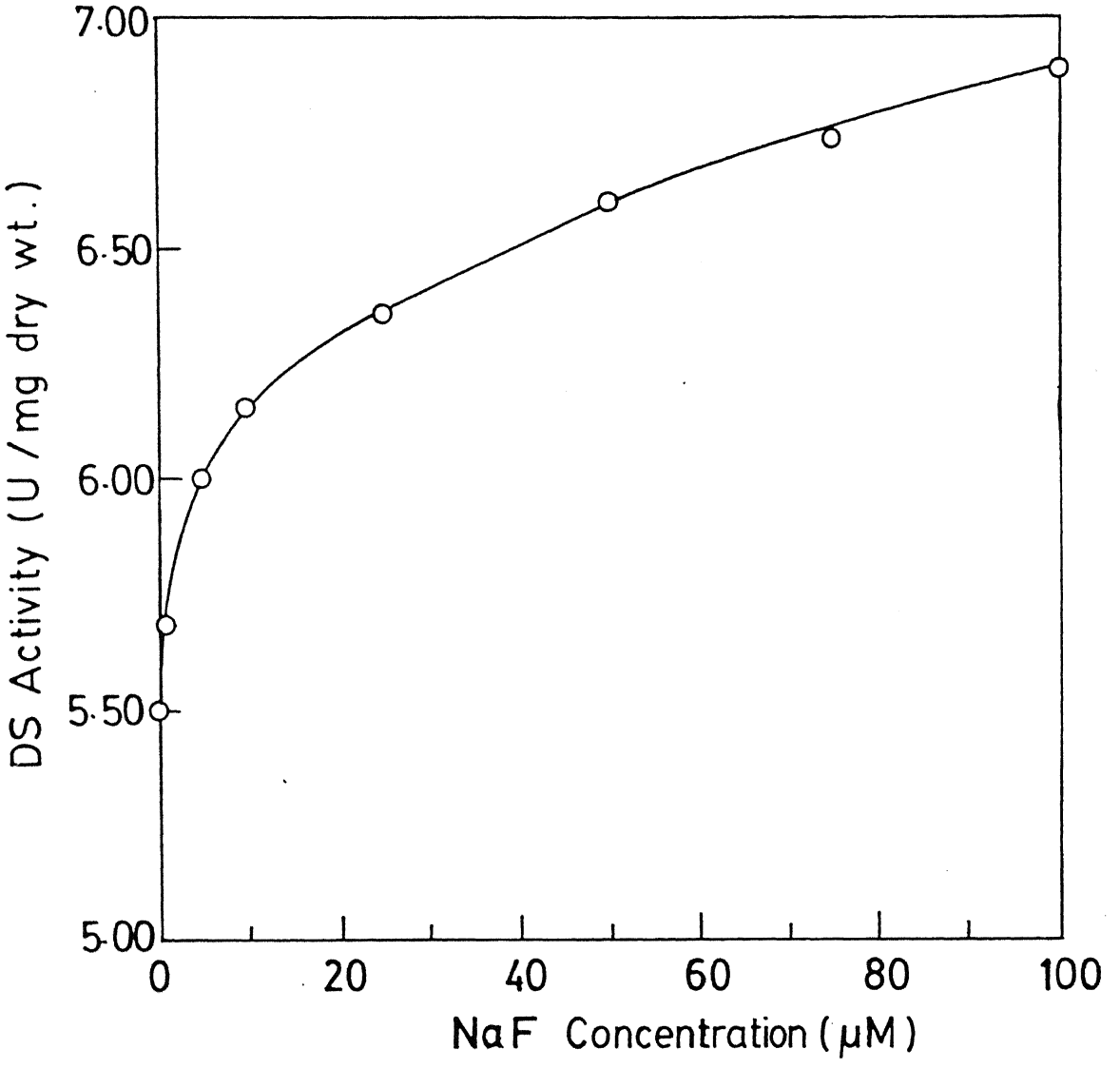


Figure II.14. Effect of NaF present in medium on extra-cellular dextransucrase activity from *Leuconostoc mesenteroides* NRRL B-512F.

II.3.5 Effect of pulse feed of sucrose on enzyme production

A time course of the pulse fed fermentation is shown in Fig. II.15. After 22 h of incubation, when the sucrose level decreased to 0.2 mg/ml, a pulse of (50 ml containing 20 g of sucrose and 20 g of dipotassium hydrogen orthophosphate) was added aseptically. As the pulse was added the enzyme activity began to rise. Two more pulses of sucrose were given at 4h interval. The pH profile of the fermenting medium paralleled with the decrease in sucrose concentration. With each addition of sucrose pulse the activity of synthesized enzyme increased. After the addition of third pulse, the enzyme activity reached to 8.5 U/ml (Fig. II.15). These observations showed that the pulse addition of sucrose and K_2HPO_4 almost gave similar results as reported by Monsan *et al.* [19] using fed-batch reactor.

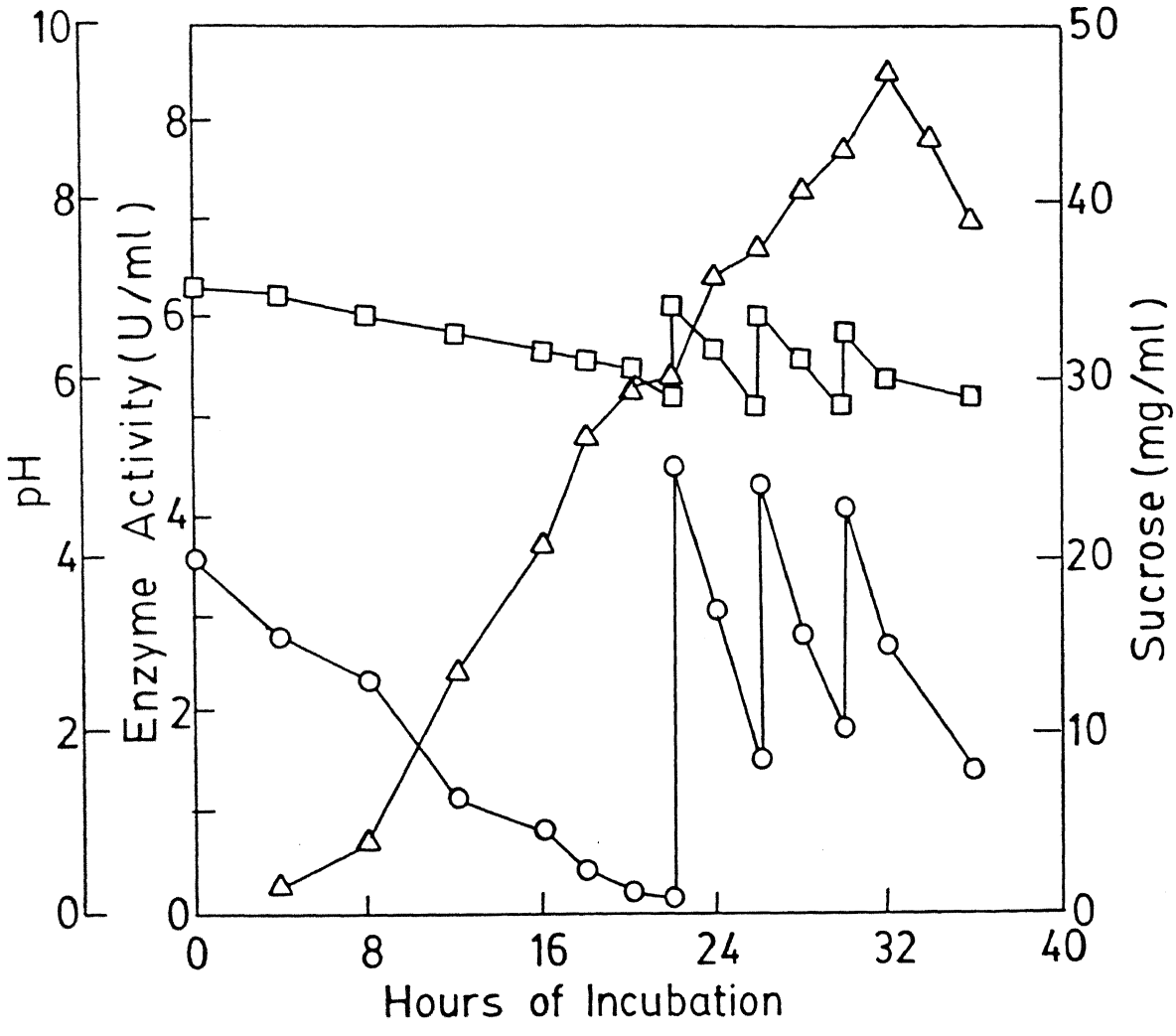


Figure II.15. Effect of pulse feed of sucrose on production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Variation of enzyme activity (Δ), pH (□) and sucrose (O) with time of incubation are shown.

II.4 SUMMARY

Modified *Lactobacillus* MRS was found to be the most suitable medium for maintenance and propagation of *Leuconostoc mesenteroides* NRRL B-512F, as it gave higher enzyme yield as compared to the other maintenance media, when the culture was transferred to EPM. This showed that the dextransucrase activity of *Leuconostoc mesenteroides* NRRL B-512F was to a large extent governed by the medium in which the culture was maintained.

The conditions for dextransucrase production vary from species to species [10,43,44] and often tend to be strain specific. The dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F is affected by temperature, pH and other culture conditions. The conventional method of production of dextran involves fermentation of dextransucrase producing microorganisms under non-aerated conditions in sucrose-rich media containing limited quantities of other nutrients. Alternatively, the dextran can be produced by a two step process involving a separate enzyme production stage followed by dextran biosynthesis stage. In the first stage, enzyme production can be optimized at conditions suitable for its elaboration. The present studies have shown that the optimum temperature of 23°C and still flask culture were found to be the most suitable conditions for production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. These conditions

can be employed for the large scale production of enzyme. The resulting enzyme can then be purified and used for synthesis of the dextran from sucrose.

The effects of certain nutrients on dextransucrase production were studied. An increase in concentration of sucrose to 4% resulted in the increase of activity of dextransucrase. Higher enzyme yields were obtained at low yeast extract and high phosphate concentration. The presence of peptone and beef extract in the medium in addition to 2% yeast extract resulted in an increased production of dextransucrase. The enzyme activity increased by approximately 30%, by both peptone (1%) and beef extract (2%). Addition of Tween 80 to EPM enhanced the production of dextransucrase and the activity was increased by 25%. Magnesium ions (50 μM) stimulated marginally (1.1 fold), the activity of dextransucrase. Sodium fluoride enhanced the activity of dextransucrase by 25% at 100 μM concentration.

The studies have amply indicated that it is imperative to identify ideal maintenance condition and growth requirements of an organism for maximum exploitation of its attributes. The present investigations carried out attain importance in the process optimization for industrial scale production of dextransucrase.

REFERENCES

1. Neely, W.B. and Nott, J., (1962) Biochemistry 1, 1136-1140.
2. Stacey, M. and Youd, F.R., (1938) Biochem. J., 32, 1943-1945.
3. Hehre, E.J., (1941) Science, 93, 237-238.
4. Stacey, M., (1942) Enzymatic production of Bacterial polysaccharides. Nature, 149, 639.
5. Hehre, E.J., (1946) J. Biol. Chem., 163, 221-223.
6. Jeanes, A., Wilham, C.A. and Miers, J.C., (1948) J. Biol. Chem., 176, 603-615.
7. Hehre, E.J., (1955) Methods Enzymol., 1, 178-184.
8. DeMan, J.C., Rogosa, M. and Sharpe, M.E., (1960) J. Appl. Bacteriol., 23, 130-135.
9. Jeanes, A., (1965) Methods Carbohydr. Chem., 5, 118-126.
10. Jeanes, A., (1965) Methods Carbohydr. Chem., 5, 127-132.
11. Lawford, G.R., Kligerman, A., Williams, T. and Lawford, H.G., (1979) Biotechnol. Bioeng., 21, 1121-1131.
12. Bhatnagar, R., Bhatnagar, N. and Prabhu, K.A., (1985) IRCS Med. Sci., 13, 129-130.
13. El-Sayed, A.H.M.M., Mahmoud, W.M. and Coughlin, R.W., (1990) Biotechnol. Bioeng., 36, 338-345.
14. Tsuchiya, H.M., Koepsell, H.J., Corman, J., Bryant, G., Bogard, M.O., Feger, V.H. and Jackson, R.W., (1952) J. Bacteriol. 64, 521-526.

15. Jeanes, A., Wilham, C.A., Tsuchiya, H.M. and Haynes, W.C., (1957) Arch. Biochem. Biophys. 71: 293-302.

16. Koepsell, H.J. and Tsuchiya, H.M., (1952) J. Bacteriol. 63, 293-295.

17. Robyt, J.F. and Walseth, T.F., (1979) Carbohydr. Res. 68, 95-111.

18. Kaboli, H. and Reilly, P.J., (1980) Biotechnol. Bioeng. 22, 1055-1069.

19. Monsan, P., Paul, F., Auriol, D. and Lopez, A., (1987) dextransucrase. Methods Enzymol. 136, 239-254.

20. Barker, P.E. and Ajongwen, N.J., (1991) Biotechnol. Bioeng. 37, 703-707.

21. Pennell, R.D. and Barker, P.E., (1992) J. Chem. Technol. Biotechnol. 53, 21-27.

22. Ajongwen, N.J. and Barker, P.E., (1993) J. Chem. Technol. Biotechnol. 56, 113-118.

23. Brown, D.E. and McAvoy, A., (1990) J. Chem. Tech. Biotechnol. 48, 405-414.

24. Rhee, S.H. and Lee, C.H., (1991) J. Microbiol. Biotechnol. 1, 176-181.

25. Kobayashi, M. and Matsuda, K., (1980) Biochim. Biophys. Acta 614, 46-62.

26. Miller, A.W., Eklund, S.H. and Robyt, J.F., (1986) Carbohydr. Res. 147, 119-133.

27. Yamashita, Y. and Takehara, T. (1989) Microbios 60, 177-182.

28. Shimura, N. and Onisi, M. (1978) J. Dent. Res. 57, 928-931.

29. Sato, M., Tsuchiya, H., Kato, M., Yamamoto, K., Nakazato, G., Tagaki, G. and Namikawa, I. (1989) Int. J. Biochem. 21, 751-754.

30. Sumner, J.B. and Sisler, E.B., (1944) Arch. Biochem. 4, 433-436.

31. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., (1951) J. Biol. Chem., 193, 265-275.

32. Goyal, A. and Katiyar, S.S., (1994). J. Microbiol. Methods 20, 225-231.

33. Kobayashi, M. and Matsuda, K. (1975) Biochim. Biophys. Acta, 397, 69-79.

34. Montville, T.J., Cooney, C.L. and Sinskey, A.J. (1977) Infect. Immun., 18, 629-635.

35. Montville, T.J., Cooney, C.L. and Synskey, A.J., (1978) Adv. Appl. Microbiol., 24, 55-84.

36. Mukasa, H., Shimamura, A. and Tsumori, H. (1989) J. Gen. Microbiol., 135, 2055-2063.

37. Hamada, S., Horikoshi, T., Okahashi, N. and Koga, T. (1989) J. Gen. Microbiol., 135, 335-344.

38. Alsop, R.M. (1983) Industrial production of dextrans. Prog. Ind. Microbiol. 18, 1-44.

39. Tanriseven, A. and Robyt, J,F. (1993) Carbohydr. Res. 245, 97-104.

40. Mizutani, N., Yamada, M., Takayama, K. and Shoda, M. (1994) J. Ferm. Bioeng. 77, 248-251.

41. Veljkovic, V.B., Lazic, M.L. and Skala, D.U. (1988) Enzyme Microb. Technol., 10, 686-688.

42. Umesaki, Y., Kawai, Y. and Mutai, M. (1977) Appl. Env. Microbiol. 34, 115-119.

43. Wittenberger, C.L., Beaman, A.J. and Lee, L.N. (1978) J. Bacteriol., 113, 231-239.

44. Castillo, E., Iturbe, F., Lopez-Munguia, A., Pelenc, V., Paul, F. and Monsan, P. (1992) Ann. N. Y. Acd. Sci. 672, 425-430.

45. Yokoyama, I., Kobayashi, M. and Matsuda, K. (1985) Agric. Biol. Chem., 49, 501-507.

CHAPTER III

PURIFICATION, CHARACTERIZATION AND PROPERTIES OF DEXTRANSUCRASE

III.1 INTRODUCTION

A series of techniques have been used for the purification of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase [1-10]. Several workers have reported that dextransucrase exists in either single or multiple forms having molecular weights in the range 64,000 - 245,000 [2,6,8,10-12]. The enzyme remains in an aggregated form in presence of dextran resulting in high molecular weight. High molecular weight proteins have been purified by precipitation using the nonionic hydrophilic polymer polyethylene glycol (PEG) [13,14]. Dextransucrase from *Streptococcus mutans* was purified by PEG precipitation [15]. However there are only a few reports in the literature on *Leuconostoc mesenteroides* NRRL B-512F dextransucrase precipitation by PEG [16,17]. In the present study a simple and effective method has been developed for the purification of

dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by fractionation with PEG of different molecular weights.

Extracellular dextransucrase has also been purified by phase-partition method [5,7]. Phase-partition occurs between dextran and polyethylene glycol (PEG). Addition of PEG solution to a dextran-rich aqueous solution, leads to the appearance of two phases; the top phase being rich in PEG while the bottom one is rich in dextran. Dextransucrase preferentially goes in to the dextran rich phase. In the present study dextransucrase was also purified by phase partitioning method using PEG of different molecular weights.

There are many reports on the properties of dextransucrase [1,3,11,12,18-21]. The enzyme can be stabilized by various agents [1,12,18]. Calcium ions have been shown to affect activity as well as the stability of dextransucrase [1,3,18]. The loss of activity could be prevented by adding calcium ions but higher concentration of calcium acted as a competitive inhibitor [20]. The dextransucrase could be stabilized against activity losses by the addition of low concentrations of non-ionic polymers like dextran, PEG 20,000 or non-ionic detergents like tween 80 and triton X100 [18]. In the present study effect of alternative stabilizers were also studied.

There are few reports on the amino acid composition of dextransucrase from *Streptococcus mutans* [23,24] and

Streptococcus sanguis [25]. However, the amino acid composition has not been reported for dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. The amino acid analysis of the purified dextransucrase was carried out using acid hydrolysis method.

III.2 MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides NRRL B-512F was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Ingredients for enzyme production media were from Hi-Media Pvt. Ltd., India. PEG 400 and PEG 600 were from BDH, India. PEG 4000 and PEG 6000 were from SD Fine Chem., India. 3,5-Dinitrosalicylic acid (DNS) was purchased from Fluka, Switzerland. Folin's reagent for protein determination, PEG 8000, dextran (Average molecular weight, 162,000), as well as the reagents used for gel electrophoresis were purchased from Sigma Chem. Co. and Bio-Rad Lab., Richmond, USA. All other chemicals used were of highest purity grade available commercially.

III.2.1 Production of dextransucrase

Leuconostoc mesenteroides NRRL B-512F was grown in sucrose medium as described in Chapter II, Section II.2.2.

III.2.2 Enzyme activity assay

The dextransucrase assay was performed at 30°C in 0.2 M sodium acetate buffer (pH 5.2). Activity was determined by measuring the production rate of reducing sugar. The reaction mixture (3.0 ml) contained 0.5 ml of 1.75 M sucrose in 0.2 M acetate buffer (pH 5.2) and 0.5 ml of enzyme solution. The reaction mixture was incubated at 30°C for 1 h. Aliquots (0.1-0.2 ml), from the reaction mixture, were taken in 3.0 ml DNS reagent and the amount of reducing sugar was determined by the method described by Sumner and Sisler [26]. Details of analysis of reducing sugar are given in Chapter II, Section II.2.3. One unit of dextransucrase activity is defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute at 30°C and pH 5.2.

III.2.3 Protein determination

Protein concentration was estimated by the method of Lowry *et al.* [27]. The details are given in Chapter II, Section II.2.

III.2.4 Fractionation of dextransucrase

The fractionation was carried out by the method as described earlier [10]. Cells were removed from culture broth by centrifugation at 10,000 rpm for 10 min at 4°C. Unless stated otherwise, all operations were carried out at 4°C. 50 ml

of crude dextransucrase with 1.4 U/ml activity was treated with PEG 400 and PEG 600 to give final (v/v) percent compositions of 33, 40, 50, 55 and 60, respectively. The required amount of PEG was added to the supernatant slowly with constant stirring. Similarly, to 50 ml crude dextransucrase were added PEG 4000 and PEG 6000 to give (w/v) percent compositions 20, 30, 40 and 50. The enzyme was allowed to precipitate out at 4°C for 24 h. The mixture was centrifuged at 12,000 rpm for 30 min at 4°C. The enzyme pellet was suspended in 10 ml of 0.2 M acetate buffer (pH 5.2) at every stage. In a typical experiment, four successive precipitation steps were performed on crude enzyme with 33% (v/v) PEG 400, and the precipitate obtained was dissolved in 10 ml sodium acetate buffer (pH 5.2).

III.2.5 Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the standard method of Laemmli [28] with modifications given below. Gel slabs of size 138×130×2 mm contained 7.5% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel. The protein samples were prepared in 0.0625 M Tris HCl buffer (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue. Electrophoresis was carried out with a current of 2 mA per lane. The protein bands were

fixed with solution containing acetic acid (5%, v/v) and (10%, v/v) methanol for 15 minutes, stained for 1 h with 0.25% (w/v) coomassie brilliant blue, and destained by repeated washing in 7% (v/v) acetic acid. The dextransucrase activity in the gel was detected by incubating a parallel gel in a solution containing 5% sucrose in 0.2 M sodium acetate buffer (pH 5.2) at 30°C for 12 h. The location of activity was visualized by means of periodic acid schiff (PAS) reaction [29].

III.2.6 Purification of dextransucrase by phase-partition

In the cell free extract, the dextran concentration was very low and it was not possible to separate PEG-dextran phase. Some amount of dextran was generated in the cell free extract. To the crude enzyme (50 ml), 5% sucrose was added and incubated at 4°C for 4 h, after which, the viscous extract was subjected to phase-separation. A 50% (w/v) PEG 6000 in water was slowly added while stirring until the turbidity appeared and then cooled at 4°C in a water bath. The final percentage of PEG 6000 in crude extract was 5%. For phase separation by PEG 400, 50 ml of crude enzyme was subjected to dextran generation by adding 5% sucrose as described earlier. The PEG 400 was added to dextran rich extract till it turned turbid and final concentration of PEG 400 was 25% (v/v).

After the phase-partition, the system was centrifuged at 10,000 rpm for 20 minutes at 4°C. The PEG rich top layer was discarded while the dextran rich bottom layer was analyzed for enzyme activity and subjected to further purification by repeating the process three times. Each time the volume of the bottom phase was adjusted to the initial volume of 50 ml using 0.2 M sodium acetate buffer (pH 5.2).

III.2.7 Effect of stabilizers on dextransucrase

The purified enzyme (0.5 mg protein/ml) was incubated at 30°C, in the presence of low concentrations of non-ionic polymer, dextran (Av. mol. wt. 162,000) (10 µg/ml) and polyethylene glycol 8000 (10 µg/ml), non-ionic detergent, tween-80 (10 µg/ml) and glycerol (0.5%, v/v). The control containing no stabilizer was also kept at 30°C. Residual activity was determined at indicated time intervals by taking 0.5 ml aliquot of the enzyme. The stabilization of dextransucrase was also observed for two weeks at 0°C using the same stabilizers. Residual activity was determined at indicated time intervals by taking an aliquot (0.5 ml) from the incubation mixture.

III.2.8. Amino acid analysis of dextransucrase

The amino acid composition of purified dextransucrase [10] was determined by hydrolyzing the lyophilized enzyme (10 mg protein) with 6 N HCl under nitrogen in sealed tubes at 110°C for 24 h. After hydrolysis, HCl was removed under vacuum and the residue thus obtained was dissolved in sodium citrate buffer (pH 2.2), filtered and applied on a column of amino acid analyzer after necessary dilution. The analysis was carried out on amino acid analyzer (Model, LKB 4101, Sweden) according to the procedure of Salnikow *et al.* [30].

The column was packed with Ultrapack II, the cation exchange resin. The column temperature was 50°C for first 45 min and 70°C till the end. A 0.2 M sodium citrate buffer of pH 3.25 was used for first 10 min followed by pH 4.25 for next 45 min and finally pH 6.45 till the end of analysis. Ninhydrin in ethylene diglycol solution containing sodium citrate buffer (pH 5.5) was used for color development at 100°C for 30 min. A mixture of standard LKB amino acids (50 n mol each), was used as standard. The quantification was done by calculating the peak area of a particular amino acid and comparing with the standard, according to the method of Moore and Stein [31]. Spectrophotometric estimation of tryptophan was carried out as per the method by Goodwin and Morton [32].

III.3 RESULTS AND DISCUSSION

III.3.1 Fractionation of dextransucrase

Dextransucrase could be precipitated from culture supernatant by PEG of low (400) (Fig. III.1) as well as high (6000) (Fig. III.2) molecular weights. Specific activity and percent yield of enzyme activity were compared (Table III.1). A final concentration of 33% (v/v) PEG 400 reproducibly gave dextransucrase with the highest specific activity of 8.7 U/mg of protein (Fig. III.1), with 80% yield and 15 fold purification (Table III.1) in a single step of precipitation. Low concentrations of PEG (400 & 600 <25% and 4000 & 6000 <20%) failed to precipitate the bulk of enzyme, which may be due to low dextran content (<1%) and more PEG going into the aqueous phase without interacting with the dextran associated with enzyme. It was repeatedly found that the enzyme preparations with higher dextran content (>2%) could be precipitated with even as low as 20% PEG 400 and 10% PEG 6000 compositions. The finding comes from dextran-PEG phase partitioning, where after the generation of dextran in the enzyme solution, low concentrations of PEG are used in order to prevent precipitation, thus giving two distinct phases of dextran and PEG [7]. An increase in the percentage of PEG led to a decrease in the recovery of activity with all the four PEGs (Fig. III.1 & Fig. III.2). Recovered specific activity of enzyme was found to be maximum at the

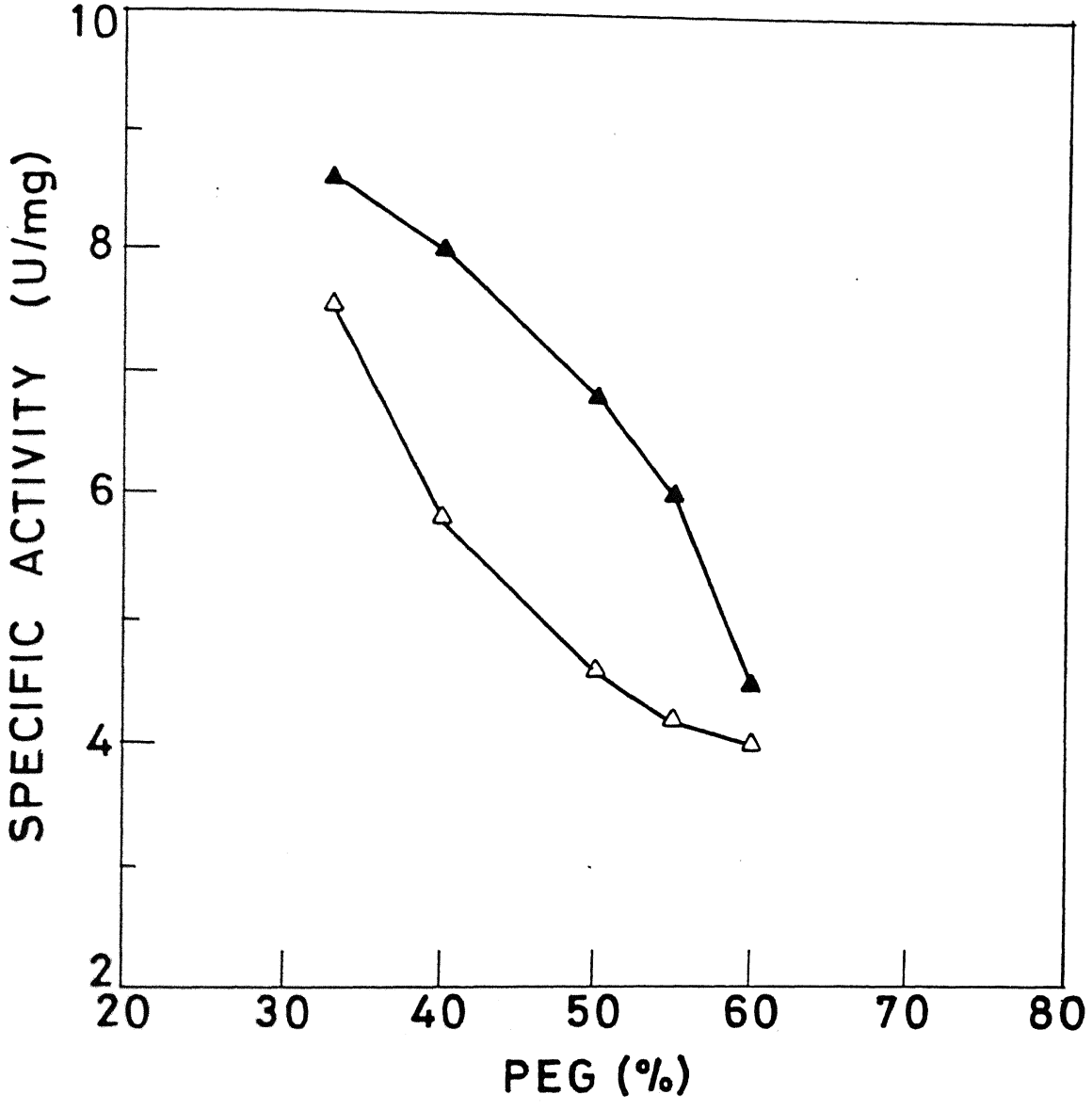


Figure III.1. Fractionation of dextransucrase by polyethylene glycol of different molecular weights. Variation of the specific activity with percent compositions of PEG 400 (▲) and PEG 600 (Δ).

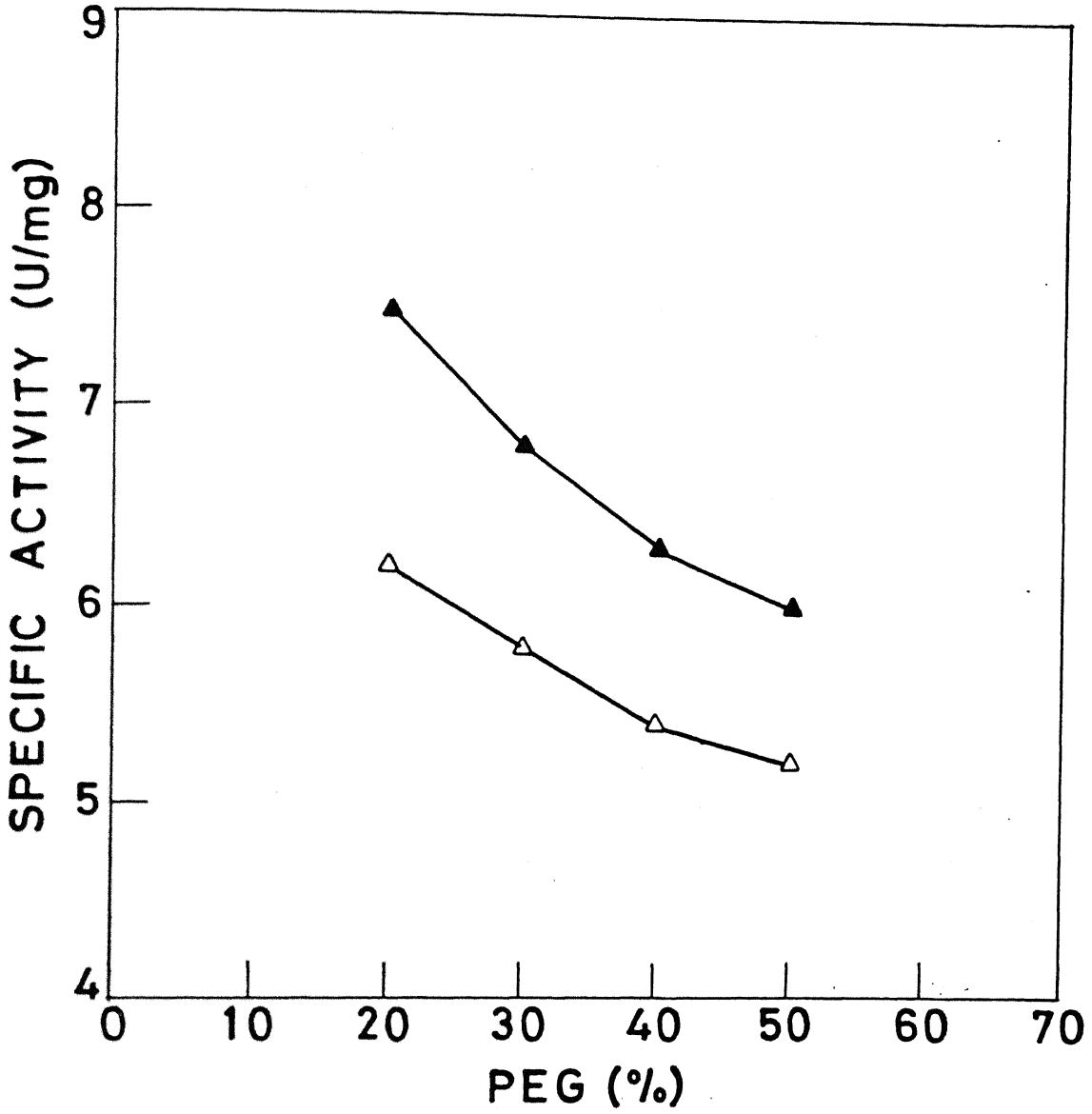


Figure III.2. Fractionation of dextransucrase by polyethylene glycol of different molecular weights. Variation of the specific activity with percent compositions of PEG 6000 (▲) and PEG 4000 (Δ).

lowest concentrations of polyethylene glycol in all the cases. Similar trend was observed with percent yield of activity in all the cases. Higher concentrations of PEG precipitated non-dextransucrase proteins also, giving impure preparations of dextransucrase, thus leading to decreased specific activity.

In four successive steps of precipitation of the crude dextransucrase (1.4 U/ml) by 33% PEG 400, maximum specific activity of 29 U/mg protein was obtained after the third step (Fig. III.3), resulting in 50 fold purification with an overall yield over 70% (Table III.2). In the subsequent fourth step, activity decreased sharply for all the concentrations of PEG 400. This may be due to the low levels of dextran left in the enzyme preparations. This is a consequence of dextran removal by PEG, and since dextran is a stabilizer for dextransucrase its removal might have caused the decrease in activity. In all steps of fractionation, specific activity obtained with 33% (v/v) concentration of PEG 400 was maximum as compared to those obtained with higher as well as lower concentrations (Fig. III.3). This suggests that 33% (v/v) PEG 400 is most suitable for fractionation and purification of dextransucrase that yields maximum enzyme activity.

Table III.1

Purification of dextransucrase by fractionation with PEG of different molecular weights

Fractionation step	Vol. ml	dextransucrase U/ml	dextransucrase Total Units	dextransucrase Overall % yield	Protein mg/ml	SA* U/mg	Fold Purification
Crude	50	1.4	70	-	2.4	0.58	-
20% PEG 4000	10	4.4	44	63	0.7	6.3	10
20% PEG 6000	10	5.9	59	85	0.8	7.4	12
33% PEG 600	10	4.2	42	60	0.55	7.6	13
33% PEG 400	10	5.6	56	80	0.65	8.7	15

*Specific activity

Table III.2

Purification of dextransucrase by fractionation in successive steps by 33% PEG 400

Fractionation step	Vol. ml	dextransucrase U/ml	dextransucrase Total Units	dextransucrase Overall % yield	Protein mg/ml	SA* U/mg	Fold Purification
Step 1	10	5.6	56	80	0.65	8.7	15
Step 2	10	5.2	52	74	0.3	17.3	30
Step 3	10	5.0	50	71	0.17	29.4	50
Step 4	10	3.2	32	45	0.16	20.0	35

*Specific activity

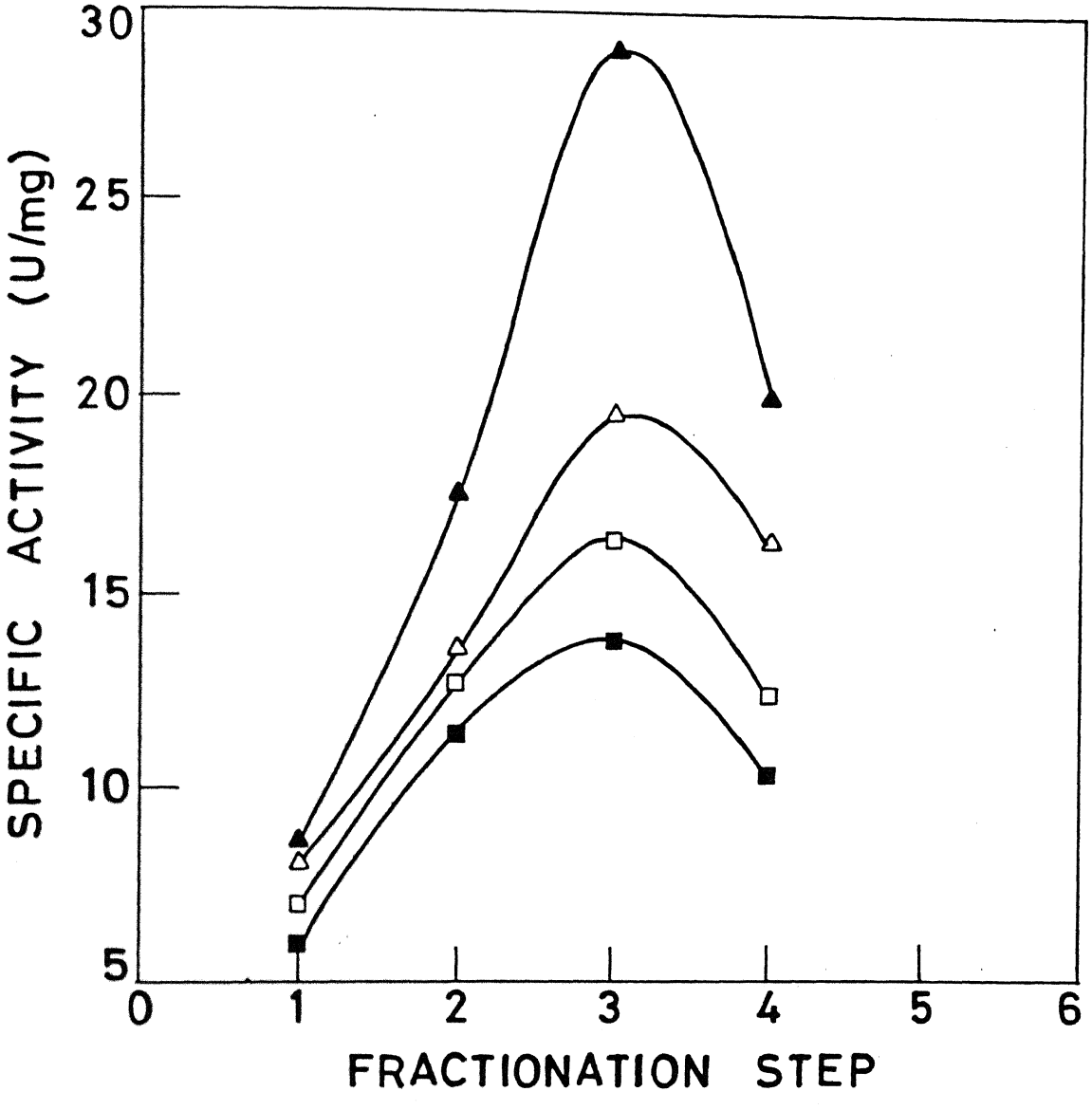


Figure III.3. Fractionation of dextransucrase by PEG 400 in four successive steps. Variation of specific activity with (v/v) percent compositions: 33% (▲); 25% (Δ); 40% (□) and 50% (■) PEG 400.

III.3.2 Characterization of purified dextransucrase

SDS-polyacrylamide gel electrophoretic analysis of dextransucrase fractionated by different concentrations of PEG 400 (Fig. III.4, Lanes B-F) showed that increase in concentration of PEG 400 precipitated additional proteins. Electrophoretic profile of culture filtrate showed over 20 proteins (Fig. III.4, Lane G). Dextransucrase exhibited multiple molecular forms having molecular weights 188,000, 126,000 and 64,000 but the molecular form with 188 kDa predominated in the crude as well as purified enzyme preparations and was found to possess greater dextran synthesizing activity as compared to other low molecular weight, molecular forms. Dextransucrase preparation obtained after three successive steps of fractionation by 33% PEG 400 was homogeneous as shown by the single band of 188 kDa on SDS-PAGE (Fig. III.4, Lane H).

III.3.3 Purification of dextransucrase by phase-partitioning

Table III.3 summarizes the results of dextransucrase purification by phase partitioning using PEG 400. In three successive steps of phase-partition a specific activity of 23.8 U/mg protein was obtained, after the third step, resulting in 30 fold purification with an overall percent yield of 46. The protein recovery was 1.5 %. Table VI.4 shows that the phase

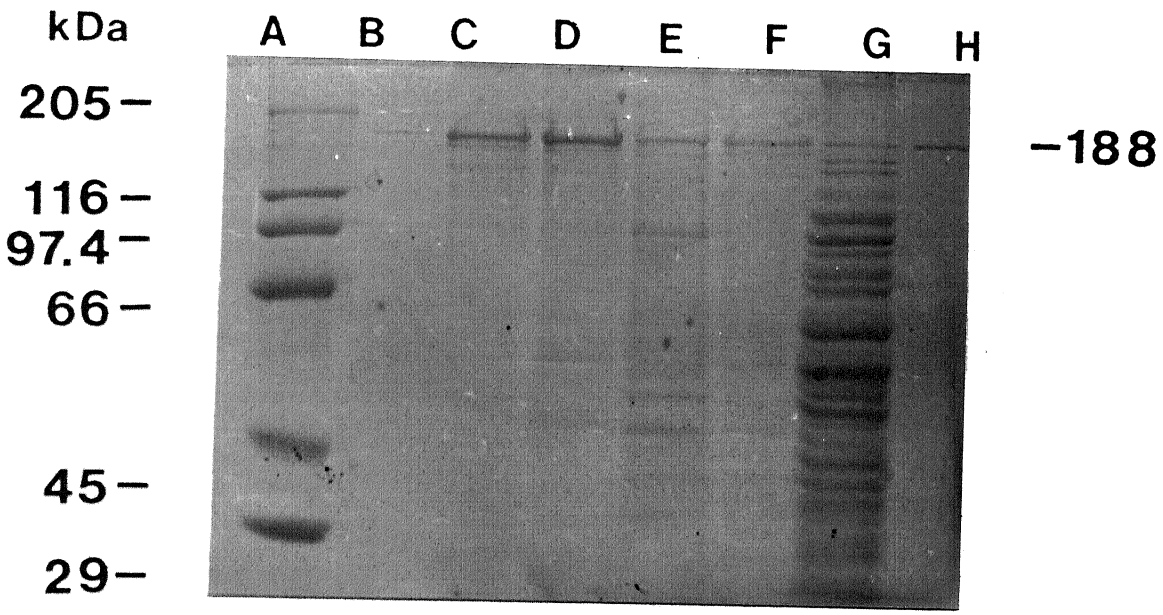


Figure III.4. SDS-polyacrylamide gel electrophoresis of dextransucrase fractionated at various concentrations of PEG 400. Molecular weight standards were (Lane A) myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase B (97.4 kDa); bovine plasma albumin (66 kDa); ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Fractionation of dextran sucrase with PEG 400 at (v/v) concentrations 20% (Lane B); 25% (Lane C); 33% (Lane D); 40% (Lane E); 50% (Lane F); crude (Lane G) and the purified dextransucrase after three successive steps of fractionation with 33% (v/v) PEG 400 (Lane H).

partitioning by PEG 6000 resulted in 65 fold increase in the specific activity with an overall yield of 78%. The specific activities of 38.7 U/mg and 23.8 U/mg were obtained in the final step of phase partitioning by PEG 6000 and PEG 400, respectively. Repeated phase-partitions resulted in purified dextransucrase preparation. The purified enzyme can be obtained with higher specific activity and yields. The continuous culture of *Leuconostoc mesenteroides* NRRL B-512F coupled with this purification technique can be used for large scale production of purified dextransucrase.

Table III.3

Purification of dextransucrase by phase-partition using PEG 400

No. of Steps	PEG 400 % (v/v)	dextransucrase			Protein	SA*	Fold Purification
		U/ml	Total Units	Overall % yield	mg/ml	U/mg	
Crude	0	1.75	87.5	-	2.20	0.89	-
Step 1	25	1.65	82.5	94	0.60	2.75	3
Step 2	15.2	0.90	45.0	52	0.11	8.20	10
Step 3	13.7	0.80	40.0	46	0.034	23.80	30

* Specific activity

Table III.4

Purification of dextransucrase by phase-partition method using PEG 6000

No. of Steps	PEG 6000 % (v/v)	dextransucrase			Protein	SA*	Fold Purification
		U/ml	Total Units	Overall % yield	mg/ml	U/mg	
Crude	0	1.40	70.0	-	2.28	0.60	-
Step 1	5	1.27	63.5	90	1.10	1.80	3
Step 2	5	1.24	62.5	89	0.285	4.4	7
Step 3	5	1.10	55.0	78	0.028	39.3	65

* Specific activity

III.3.4 Effect of stabilizers

Dextransucrase is very sensitive to temperature and pH. The purified dextransucrase exhibited maximum activity at 30°C and pH of 5.2. The effect of various stabilizers on dextransucrase was determined at 0°C and 30°C. Among four stabilizers (glycerol, tween-80, dextran and PEG 8000) used in this study, glycerol provided the maximum protection to dextransucrase against inactivation at both the temperatures. With glycerol there was 18% loss in the enzyme activity after 6 h at 30°C, while, with PEG 8000, dextran, tween-80 and no stabilizer the enzyme lost 25, 35, 50 and 70% of the activity, respectively (Fig. III.5). Studies on the effect of the same

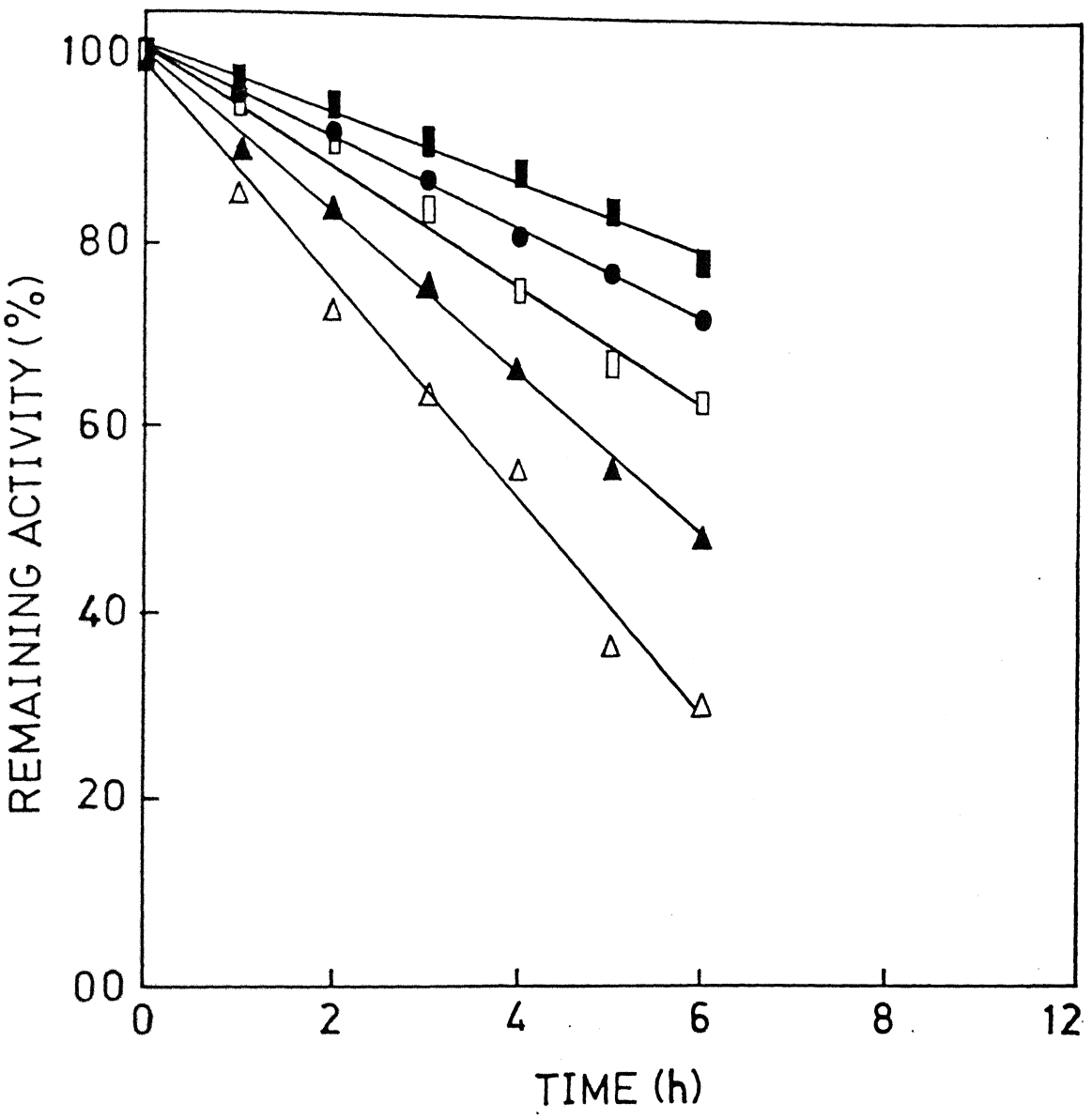


Figure III.5. Effect of stabilizers against activity losses of dextransucrase at 30°C. The enzyme (0.5 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with glycerol 0.5%, (■); PEG 8000, 10 µg/ml (●); dextran, 10 µg/ml (□); tween-80, 0.5% (▲) and control with no stabilizer (Δ).

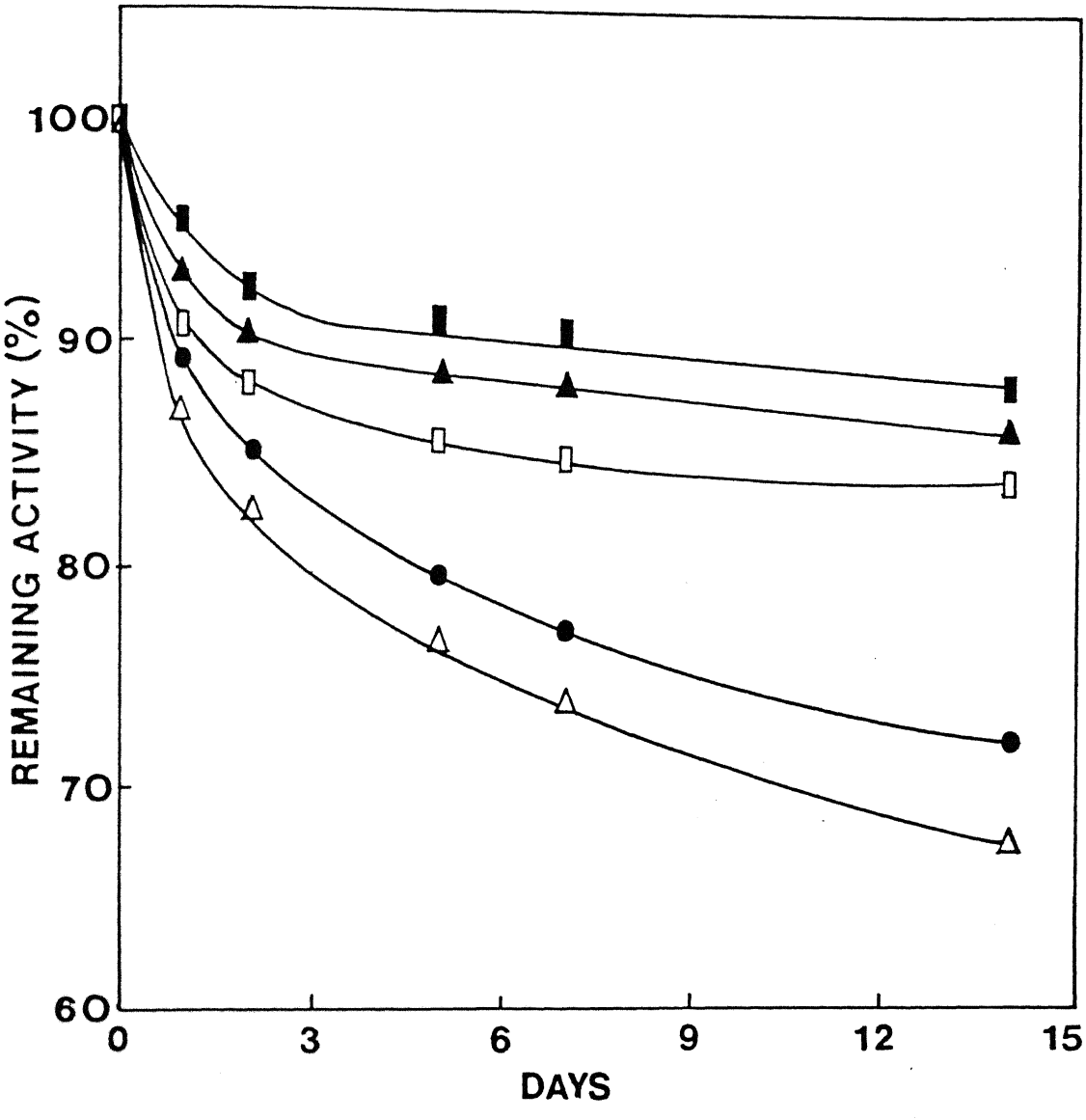


Figure III.6. Effect of stabilizers against activity losses of dextransucrase at 0°C. The enzyme (0.5 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with glycerol, 0.5% (■); PEG 8000, 10 μg/ml (▲); dextran, 10 μg/ml (□); tween-80, 0.5% (●) and control with no stabilizer (Δ).

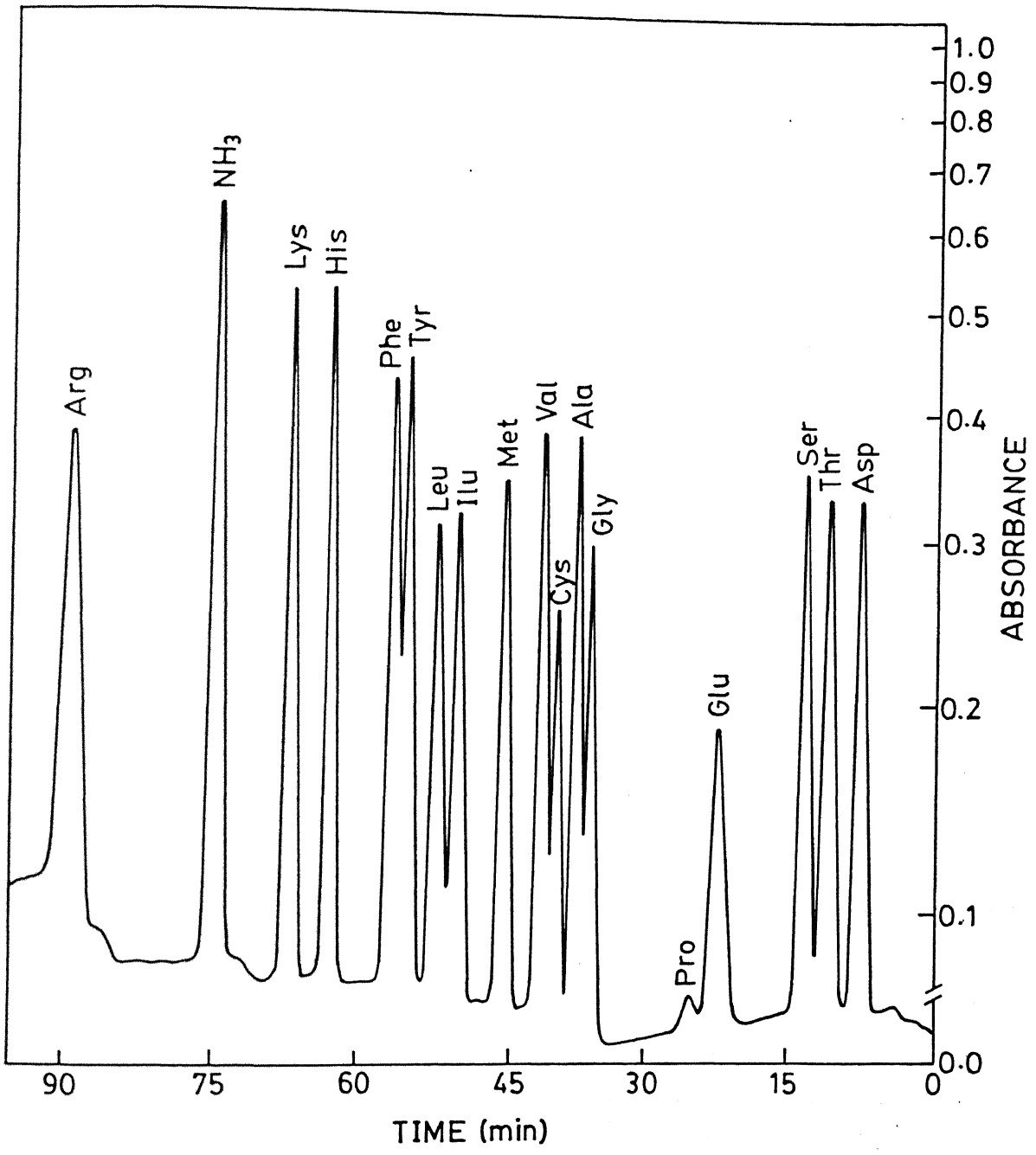


Figure III.7. Chromatogram showing the analysis of standard mixture containing 50 nmol of each amino acid. The absorbance was monitored at 440 and 570 nm.

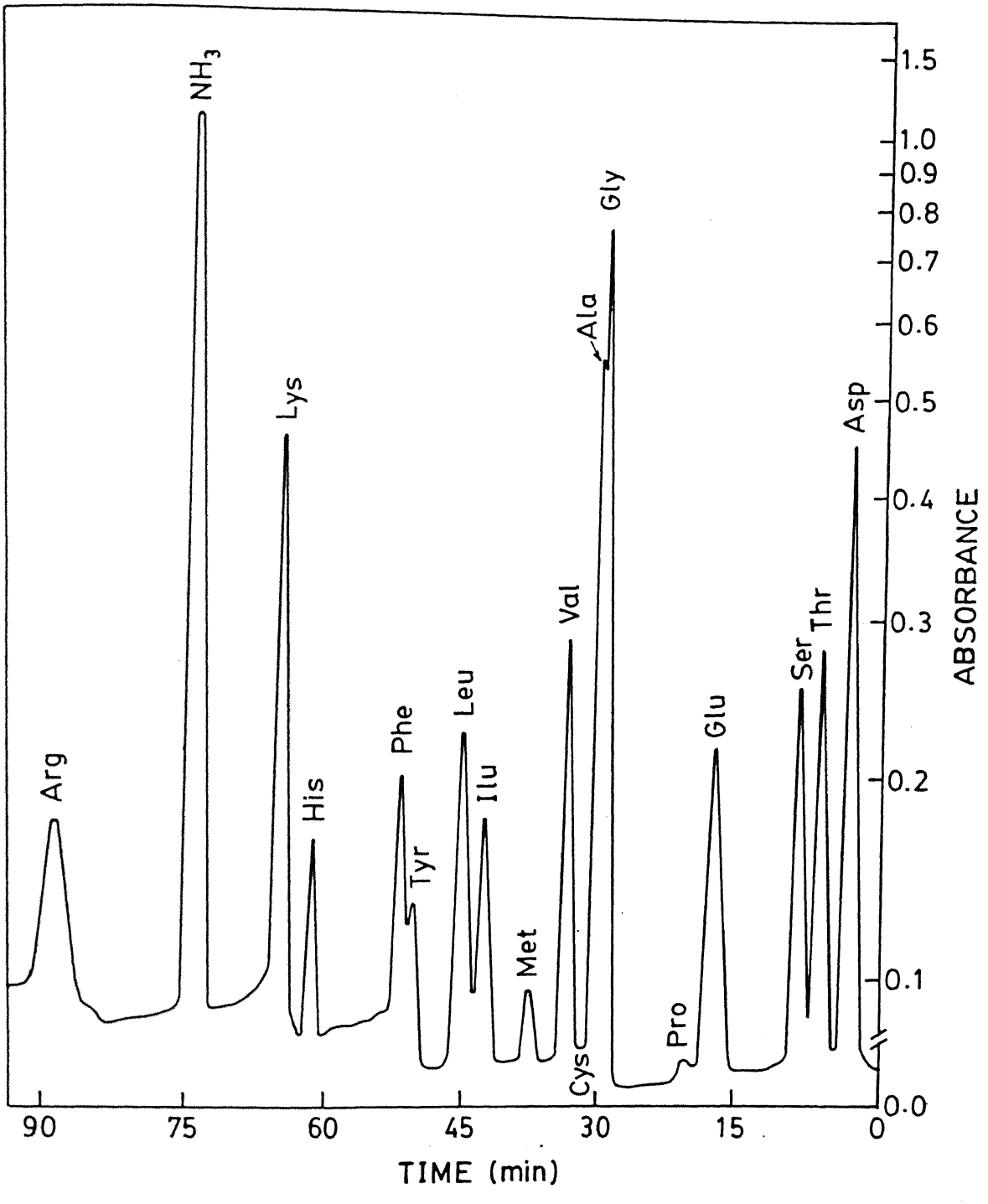


Figure III.8. Chromatogram showing the amino acid analysis of dextransucrase. Protein hydrolysate was prepared by acid hydrolysis as described in "Materials and Methods". The

Table III.5

Amino acid composition of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F

Amino acid	Average number of residues per molecule of enzyme of mol. wt. 188,000
Aspartic acid	150 (±4)
Threonine	94 (±2)
Serine	82 (±2)
Glutamic acid	113 (±3)
Proline	41 (±2)
Glycine	428 (±7)
alanine	221 (±5)
Cysteine	1.5 (±0.3)
Valine	106 (±2)
Methionine	16 (±1)
Isoleucine	63 (±2)
Leucine	91 (±2)
Tyrosine	22 (±1)
Phenylalanine	42 (±2)
Histidine	28 (±1)
Lysine	102 (±2)
Arginine	50 (±1)
Tryptophan	7 (±3)

III.4 SUMMARY

It was found that a three step fractionation with a final concentration of 33% PEG 400, reproducibly yielded a homogeneous preparation of dextransucrase, showing a single band on analysis by SDS-polyacrylamide gel electrophoresis. The PEG-fractionation method for purification of dextransucrase described in the present study is simple, inexpensive and less time consuming as compared to the other purification methods using ultrafiltration and the chromatography [2,6-8]. The overall yield of dextransucrase activity obtained after the third step of precipitation by PEG 400 was 71% (Table 1, Part B) which is significantly higher than those, reported previously [2,6,8]. Thus the method provides a useful means of producing the purified enzyme with high yield of protein and activity. The method described above can be scaled up to deal with large volumes and could be of general use for preparation of dextransucrase. The studies have further shown that evolving a comprehensive purification methodology relying on fractionation by PEG requires a thorough understanding of protein-dextran, protein-PEG and dextran-PEG interaction in selective use of the fractionation process.

The purification of dextransucrase by phase-partitioning using PEG 400 and PEG 6000 described earlier showed that repeated phase-partition by PEG 6000 leads to a degree of

purity much greater than that obtained by PEG 400. The process is quick and easy and allows the rapid concentration of crude enzyme having a positive effect on the enzyme stability.

The amino acid composition of dextransucrase showed that there is only one cysteine residue per enzyme molecule. This illustrates that its tertiary structure must be solely dependent on other types of secondary bonding such as hydrogen bonding and ionic and non polar interactions. The presence of only one cysteine residue eliminates the possibility that disulfide bonds are responsible for the retention of tertiary structure. Dextransucrase could be stabilized by additives like dextrans, tween 80, polyethylene glycols and glycerol. The methods for dextransucrase purification and its properties, attain importance, in process optimization for industrial scale production of dextran.

REFERENCES

1. Robyt, J.F. and Walseth, T.F. (1979) Carbohydr. Res. 68, 95-111.
2. Kobayashi, M. and Matsuda, K. (1980) Biochim. Biophys. Acta 614, 46-62.
3. Kaboli, H. and Reilly, P.J. (1980) Biotechnol. Bioeng., 22, 1055-1069.
4. Monsan, P. and Lopez, A. (1981) Biotechnol. Bioeng., 23, 2027-2037.
5. Paul, F., Auriol., D., Oriol, E. and Monsan, P. (1984) Ann. N. Y. Acad. Sci., 434, 267-270.
6. Miller, A.W., Eklund, S.H. and Robyt, J.F. (1986) Carbohydr. Res., 147, 119-133.
7. Monsan, P., Paul, F., Auriol, D. and Lopez, A. (1987), Methods Enzymol. 136, 235.
8. Fu, D. and Robyt, J.F. (1990) Prep. Biochem. 20, 93-106.
9. Rhee, S.H. and Lee, C.-H. (1991) J. Microbiol. Biotechnol. 1(3), 176-181.
10. Goyal, A. and Katiyar, S.S. (1994) J. Microbiol. Methods 20, 225-231.
11. Kobayashi, M. and Matsuda, K. (1986) J. Biochem. 100, 615-621.
12. Willemot, R.M., Monsan, P. and Durand, G. (1988) Ann. N. Y. Acad. Sci. 542, 169-172.

13. Honig, W. and Kula, M.R. (1976) Anal. Biochem. 72, 502-512.

14. Meikka, S.I. and Ingham, K.C. (1978) Arch. Biochem. Biophys. 191, 525-536.

15. Russell, R.R.B. (1979) FEMS Microbiol. Lett. 6, 197-199.

16. Barker, P.E., Zafar, I. and Aslop, R.M. (1987) Separations for Biotechnology, pp 127-151, ed. Verrall, M.S. and Hudson, M.J., Ellis Horwood, Chichester, UK.

17. Ajongwen, J., Akintoye, A., Barker, P.E., Ganetsos, G., and Shieh, M.T. (1993) Chem. Eng. J. 51, B43-B50.

18. Miller, A.W. and Robyt J.F. and (1984) Biochim. Biophys. Acta 785, 89-96.

19. Miller, A.W. and Robyt, J.F. (1986) Biochim. Biophys. Acta, 870, 198-203.

20. Miller, A.W. and Robyt, J.F. (1986) Biochim. Biophys. Acta 880, 32-39.

21. Kobayashi, M., Mihara, K. and Matsuda, K. (1986) Agric. Biol. Chem., 50(3), 551-556.

22. Rhee, S.H. and Lee, C.-H. (1991) J. Microbiol. Biotechnol. 1(3), 176-181.

23. Shimamura, A., Tsumori, H. and Mukasa, H. (1982) Biochim. Biophys. Acta, 702, 72-80.

24. Mukasa, H., Shimamura, A. and Tsumori, H. (1989) J. Gen. Microbiol., 135(7), 2055-2063.

25. Grahame, D.A. and Mayer, R.M. (1985) Carbohydr. Res., 142, 285-298.

26. Sumner, J.B. and Sisler, E.B. (1944) Arch. Biochem. 4, 333-336.

27. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

28. Laemmli, U.K. (1970) Nature 227, 680-685.

29. Zacharius, R.M. and Zell, T.E. (1969) Anal. Biochem. 30, 148-152.

30. Salnikow, J., Liao, T.-H., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 1480-1488.

31. Moore, S. and Stein, W.H. (1963) Methods Enzymol. 6, 819-831.

32. Goodwin, W.T. and Morton, R.A. (1946) Biochem. J. 40, 628-632.

CHAPTER IV

ACTIVE SITE MAPPING STUDIES ON DEXTRANSUCRASE USING PYRIDOXAL 5'-PHOSPHATE AND 2,4,6-TRINITROBENZENESULPHONIC ACID

IV.1 INTRODUCTION

Leuconostoc mesenteroides NRRL B-512F produces an extra cellular enzyme dextransucrase {Sucrose: 1,6-α-D glucan 6-α-D glucosyltransferase, EC 2.4.1.5} that catalyzes the formation of dextran from sucrose.

$$\underset{\text{(SUCROSE)}}{n\ C_{12}H_{22}O_{11}} \longrightarrow \underset{\text{(DEXTRAN)}}{(C_6H_{10}O_5)_n} + \underset{\text{(FRUCTOSE)}}{n\ C_6H_{12}O_6}$$

Many reports have appeared on the purification of dextransucrase and have shown that it exists in either single or multiple forms having molecular weights in the range 64,000 - 245,000 [1-6]. We have observed that the purification

by polyethylene glycol 400 gives homogeneous and single molecular form of dextransucrase of the size 188 kDa [7].

Although extensive work has been carried out on the mechanism of dextran synthesis and structural organization of catalytic site of dextransucrase [8-15], not much information is available on the nature of amino acids at the active site. A two-site mechanism was proposed for dextran synthesis and it was shown that the two nucleophiles at the active site attack two bound sucrose molecules to give two covalent intermediates [10]. Measurement of the rate of reaction as function of pH indicates the presence of catalytically important groups with pK_a values of 4.5 and 7.5 [11]. Chemical modification of the enzyme with carbodiimdes and glycine methyl ester results in non-specific modification of carboxylates and loss of the activity [12]. An active site peptide containing covalently bound glucosyl group to aspartic acid was isolated from dextransucrases of *Streptococcus sobrinus* [13]. Further, a mechanism was also proposed in which a residue aspartic acid, stabilizes the carbonium ion in equilibrium with the covalent glucosyl-enzyme complex and another residue, histidine facilitates the departure of fructose by donating a proton to the glucoside oxygen. Later, by chemical modification studies using diethyl pyrocarbonate it was shown that the two essential

histidine residues are present at the active site and was further proposed that the imidazolium groups of histidine residues donate protons to the leaving D-fructosyl moieties and the resulting imidazole groups facilitate the formation of α-(1⟶6)-glucosidic linkage by abstracting protons from the C-6-OH groups [14].

Lysine has been identified as the catalytic residue in various enzyme systems using the lysine-specific reagents, pyridoxal 5′-phosphate (PLP) [16-21] and 2,4,6-trinitrobenzene-sulphonic acid (TNBS) [22,23]. The present study has shown for the first time, the presence of an essential lysine residue at the active site of dextransucrase by chemically modifying the enzyme with PLP [21] and TNBS.

IV.2 MATERIALS AND METHODS

Materials

Pyridoxal-5′ phosphate (PLP), 2,4,6-trinitrobenzenesulphonic acid (TNBS), L-lysine, dextranase, ethylene diaminetetra-acetic acid (EDTA) and dextran (Average molecular weight, 162,000) were obtained from Sigma Chemical Company, (St. Louis, USA). All other chemicals were of highest purity grade, available commercially.

Methods

IV.2.1 Analytical procedures

IV.2.1.1 *Preparation of reagents for reducing sugar estimation*

The reagents for estimation of reducing sugar were prepared by the method of Nelson [24] and Somogyi [25].

Reagent A : Sodium carbonate anhydrous (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g) and sodium sulfate anhydrous (200 g) dissolved in water and the volume made upto 1 l. Filtered and stored at a temperature between 30-37°C.

Reagent B : 15% Copper sulfate containing one or two drops of concentrated sulfuric acid.

Reagent C : Ammonium molybdate (25 g) in 450 ml, added 21 ml of concentrated sulfuric acid mixed. To this was added sodium arsenate (3 g) dissolved in 25 ml of water and mixed. Placed in an incubator at 37°C for 24 h.

Reagent D : Prepared fresh, by mixing 25 ml of reagent A and 1 ml of reagent B.

IV.2.1.2 *Reducing sugar estimation*

To 1 ml sample containing the reducing sugar, 1 ml of reagent D was added. The solutions were mixed and heated for 20 min in boiling water bath. It was cooled in pan of cold water, and 1 ml of reagent C was then added. The color developed rapidly and completed after the evolution of carbondioxide was stopped. The mixture was diluted by adding water, making up the volume to 10 ml and absorbance measured against blank at 500 nm on UV-Visible spectrophotometer, (Shimadzu, Model UV-160A).

IV.2.2 Assay of dextransucrase activity

The dextransucrase assay was performed at 30°C in 0.2 M sodium acetate buffer (pH 5.2). The enzyme activity was determined by measuring the rate of production of reducing sugar. The assay mixture (1.0 ml) contained 10% substrate sucrose in 0.2 M acetate buffer (pH 5.2) and the enzyme solution. The reaction mixture was incubated at 30°C for 20 min. Aliquots (0.1-0.2 ml), from the reaction mixture were analyzed for reducing sugar as described in Section IV.2.1.

IV.2.3 Calculation of dextransucrase activity

The activity of enzyme is expressed as Units/ml and the specific activity as Units/mg of protein. One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 μmole of reducing sugar per minute.

The dextransucrase activity was calculated as follows:

$$\text{Enzyme activity (Units/ml)} = \frac{\Delta(A_{500}) \times C \times V}{180 \times t \times v} \quad (\mu \text{ mole/min/ml})$$

$\Delta(A_{500})$ = change in absorbance of the sample at 500 nm

C = 1 OD equivalent fructose from standard plot

V = volume of reaction mixture (ml)

t = time of reaction (min)

180 = molecular weight of fructose

v = volume of reaction mixture for reagent (ml)

IV.2.4 Enzyme preparation

Dextransucrase produced from *Leuconostoc mesenteroides* NRRL B-512F was purified by fractionation with polyethylene glycol 400 as reported previously [7]. The purified enzyme had a specific activity of 30 U mg^{-1} protein. One unit of dextransucrase activity is defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute at 30°C and pH 5.2. For

all modification experiments dextran free enzyme was used and the native dextran present in the enzyme was removed by dextranase treatment [2].

IV.2.5 Reaction of dextransucrase with pyridoxal 5'-phosphate

The enzyme (1.2 mg of protein/ml) in 0.2 M acetate buffer (pH 5.2), was incubated with specified concentrations of PLP at 30°C. The reaction of enzyme with PLP was stopped at different time intervals by transferring two parts of an aliquot of reaction mixture to eight parts of 0.2 M acetate buffer (pH 5.2) containing 10 mM L-lysine. An aliquot from the lysine mixture was assayed for the enzyme activity as described in Section IV.2.2. The time course of modification by PLP was monitored continuously by recording the change in absorbance at 425 nm [26].

IV.2.6 Reactivation of dextransucrase after inactivation by pyridoxal 5'-phosphate

Reactivation of the enzyme was achieved by 20 fold dilution of the Enzyme-PLP mixture with 0.2 M acetate buffer (pH 5.2). Aliquots were taken out at specified time intervals and the enzyme activity was determined as described in Section IV.2.2. A control without PLP was run concurrently.

IV.2.7 Spectral analysis of pyridoxal 5'-phosphate-modified dextransucrase

The enzyme (1.2 mg/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 30 mM PLP for 1 h at 30°C. The enzyme-inhibitor reaction was terminated by reduction with 50 mM sodium borohydride. The reaction mixture was kept at 0°C for 30 min and then dialyzed extensively against 0.2 M acetate buffer (pH 5.2). The resulting N^{ε}-phosphopyridoxyllysine complex was characterized by fluorescence emission spectrum with excitation at wave length, 325 nm [26].

IV.2.8 Effect of substrates on inactivation of dextransucrase by pyridoxal 5'-phosphate

For protection experiments, the enzyme (1.2 mg protein/ml, 30 U/mg) was incubated with 50 mM EDTA in 0.2 M acetate buffer (pH 5.2) at 30°C for 30 min. The substrate, sucrose was added and incubated for another 10 min at 30°C prior to the addition of 30 mM PLP. After 1h the residual activity was determined following the same procedure as described in Section IV.2.2. The assay mixture contained 50 mM of Ca^{2+} for reactivation of the enzyme. The enzyme preincubated with EDTA followed by treatment with Ca^{2+} without PLP was used as control.

The enzyme (1.2 mg protein/ml) was incubated with 50 mM and 100 mM glucose for 15 min at 30°C prior to the addition of 30 mM PLP. After 1h the residual activity was determined following the same procedure as described in Section IV.2.2. The appropriate controls without PLP and with glucose were run concurrently.

The enzyme in 0.2 M acetate buffer (pH 5.2) was incubated with 5 mg/ml of dextran (Av. mol. wt. 162,000) at 30°C for 15 min. The enzyme was then modified with 30 mM PLP. After 1h the residual activity was determined following the same procedure as described in Section IV.2.2. The activity was assayed without the removal of dextran, which has no effect on the activity [27]. A control without dextran was similarly prepared.

IV.2.9 Stoichiometry of pyridoxal 5'-phosphate binding with dextransucrase

Stoichiometric determinations were carried out by incubating the enzyme (1.2 mg/ml) with 30 mM PLP for different time intervals at 30°C. The enzyme-inhibitor reaction was terminated by reduction with 50 mM sodium borohydride in 0.2 M acetate buffer (pH 5.2). The reaction mixture was kept at 0°C for 30 min and then dialyzed extensively against 0.2 M acetate

buffer (pH 5.2). The number of mol of PLP incorporated per mol of enzyme was determined by taking the ratio of concentrations of N^{ϵ}-phosphopyridoxyllysine and the enzyme. The concentrations of N^{ϵ}-phosphopyridoxyllysine and enzyme were determined by using the absorbance coefficient of 9700 $M^{-1}cm^{-1}$ at 325 nm [26] and Lowry *et al.* method [28], respectively. The molecular weight of dextransucrase was taken as 188,000 [7].

IV.2.10 Dextransucrase inactivation studies using 2,4,6-trinitrobenzenesulphonic acid

The enzyme (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2), was incubated with specified concentrations TNBS at 30°C. The reaction of enzyme with TNBS was stopped at different time intervals by transferring two parts of an aliquot of reaction mixture to eight parts of 0.2 M acetate buffer (pH 5.2) containing 10 mM L-lysine. An aliquot from the lysine mixture was assayed for the enzyme activity as described earlier. The time course of modification by TNBS was monitored continuously by recording the change in absorbance at 367 nm [29].

IV.2.11 Quantitative measurement of reaction of 2,4,6-trinitrobenzenesulphonic acid with dextransucrase

The stoichiometry of inactivation by TNBS was determined by incubating dextransucrase (1.2 mg protein/ml) with 30 mM TNBS and modification was observed by absorbance increase at 367 nm [29]. The number of mol of TNBS incorporated per mol of enzyme was determined by taking the ratio of concentrations of trinitrophenyl derivative of amino group of lysine (ε-TNP-lysine) and the enzyme. The concentrations of ε-TNP-lysine and the enzyme were determined by using absorbance coefficient of 11,000 $M^{-1}cm^{-1}$ at 367 nm [29] and Lowry *et al.* method [28], respectively. The molecular weight of dextransucrase was taken as 188,000 [7].

IV.2.12 Amino acid analysis of dextransucrase

For determination of amino acid composition, the lyophilized enzyme (10 mg protein) was hydrolyzed with 6 N HCl under nitrogen in sealed tubes at 110°C for 24 h and analyzed with amino acid analyzer (Model, LKB 4101, Sweden) according to the procedure of Salnikow, *et al.* [30]. The details are described in Chapter III, Section III.2.8.

IV.3 RESULTS AND DISCUSSION

IV.3.1 Inactivation of dextransucrase by pyridoxal 5'-phosphate

PLP has been used over a wide range of concentrations, from 0.1 to 24 mM [16-21,31,32]. In the present investigation, the concentration of PLP required for inactivation of dextransucrase was higher than that reported for some other enzymes. The time period required for complete inactivation of dextransucrase was also longer. This could reflect the possibility that the lysyl residues at the active site are less accessible, which might be wholly, or partially, due to the presence of covalently bound dextran at the active site. The treatment of dextransucrase with PLP in concentration range 10-35 mM led to the loss of enzyme activity and nearly 80% inactivation occurred in 1h at 35 mM PLP (Fig. IV.1). Plots of log (residual activity) *versus* time of incubation were linear, indicating that the inactivation followed pseudo-first order kinetics (Fig. IV.1). A plot of the observed pseudo-first order rate constants against the concentrations of PLP gave a straight line passing through the origin (Fig. IV.1, inset), from which a second order rate constant of 0.6 $M^{-1}min^{-1}$ was obtained. It was found that even when dextransucrase was incubated with PLP for a longer period complete loss of enzyme activity was not obtained. Chen and Engel [28] demonstrated

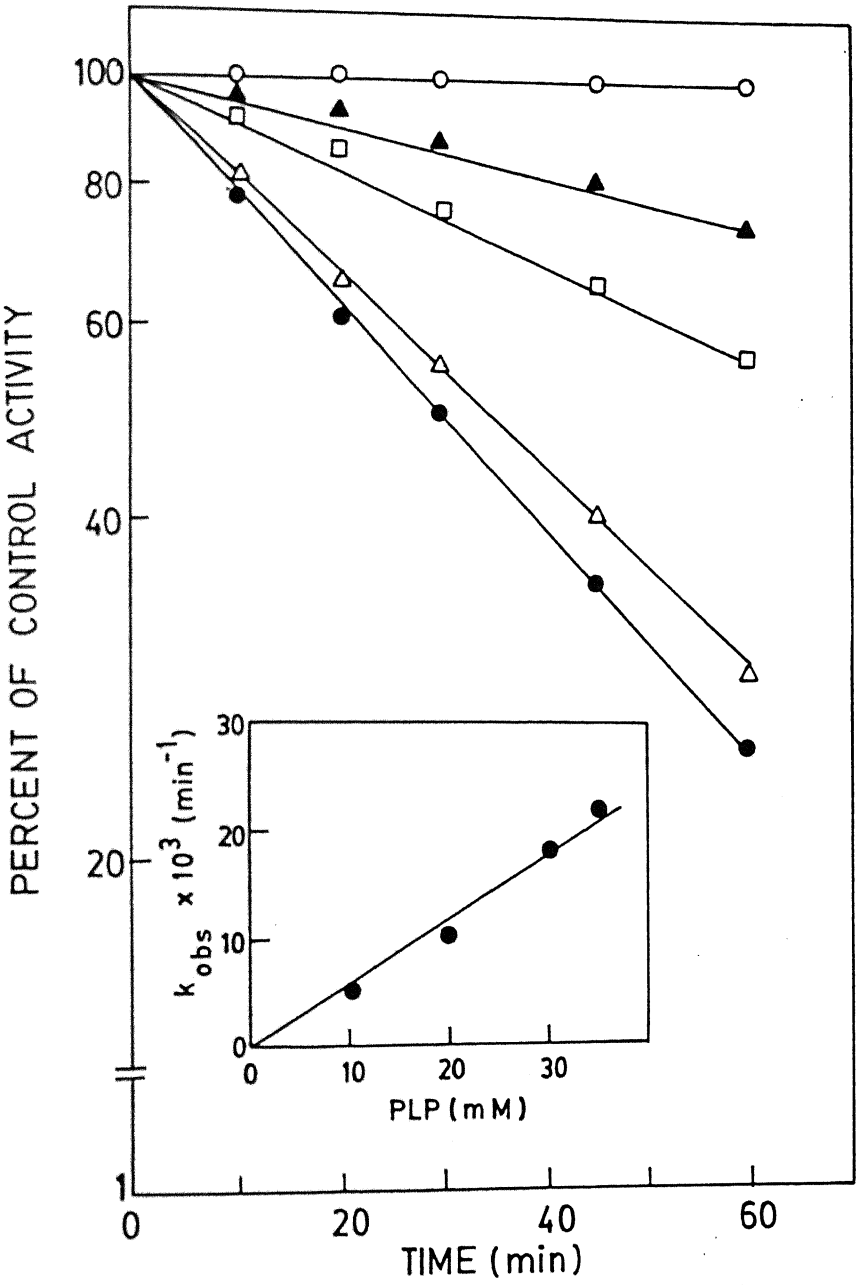


Figure IV.1. Kinetics of inactivation of dextransucrase by PLP. The enzyme (1.2 mg/ml, 30 U/mg protein) in 0.2 M acetate buffer (pH 5.2), was incubated with 0 (O); 10, (▲); 20, (□); 30, (Δ); and 35 mM (●) PLP at 30°C. Aliquots were withdrawn at indicated time intervals and the residual activity was determined as described in "Materials and Methods". Inset: a plot of observed pseudo-first order rate constant *vs* initial PLP concentrations, from which a second order rate constant was obtained.

that the residual activity of the enzyme is due to breakdown of catalytically inactive Schiff's base to free enzyme through an intermediate non covalent enzyme-PLP complex. This process can be represented in the following way,

$$E + PLP \underset{k_2}{\overset{k_1}{\leftrightarrow}} \underset{\text{noncovalent complex}}{E\text{-}PLP} \underset{k_4}{\overset{k_3}{\leftrightarrow}} \underset{\text{covalent complex}}{E'\text{-}PLP}$$

The noncovalent binding step is rapid in both directions. When the enzyme-PLP complex containing solution is diluted there is a decrease in the concentration of both enzyme as well as PLP so that E-PLP dissociates quantitatively to give back the free, active enzyme [28].

The modification with PLP led to an increase in absorbance at 425 nm which was proportional to the degree of inactivation (Fig. IV.2). The inactivation of dextransucrase was found reversible on dilution and 100, 85 and 75% of the activity could be regained when the initial concentration of PLP was 10, 20 and 30 mM, respectively (Fig. IV.3). Reactivation of the enzyme was also observed when the PLP treated enzyme was dialyzed against the 0.2 M acetate buffer (pH 5.2). However, reduction with sodium borohydride after PLP treatment rendered

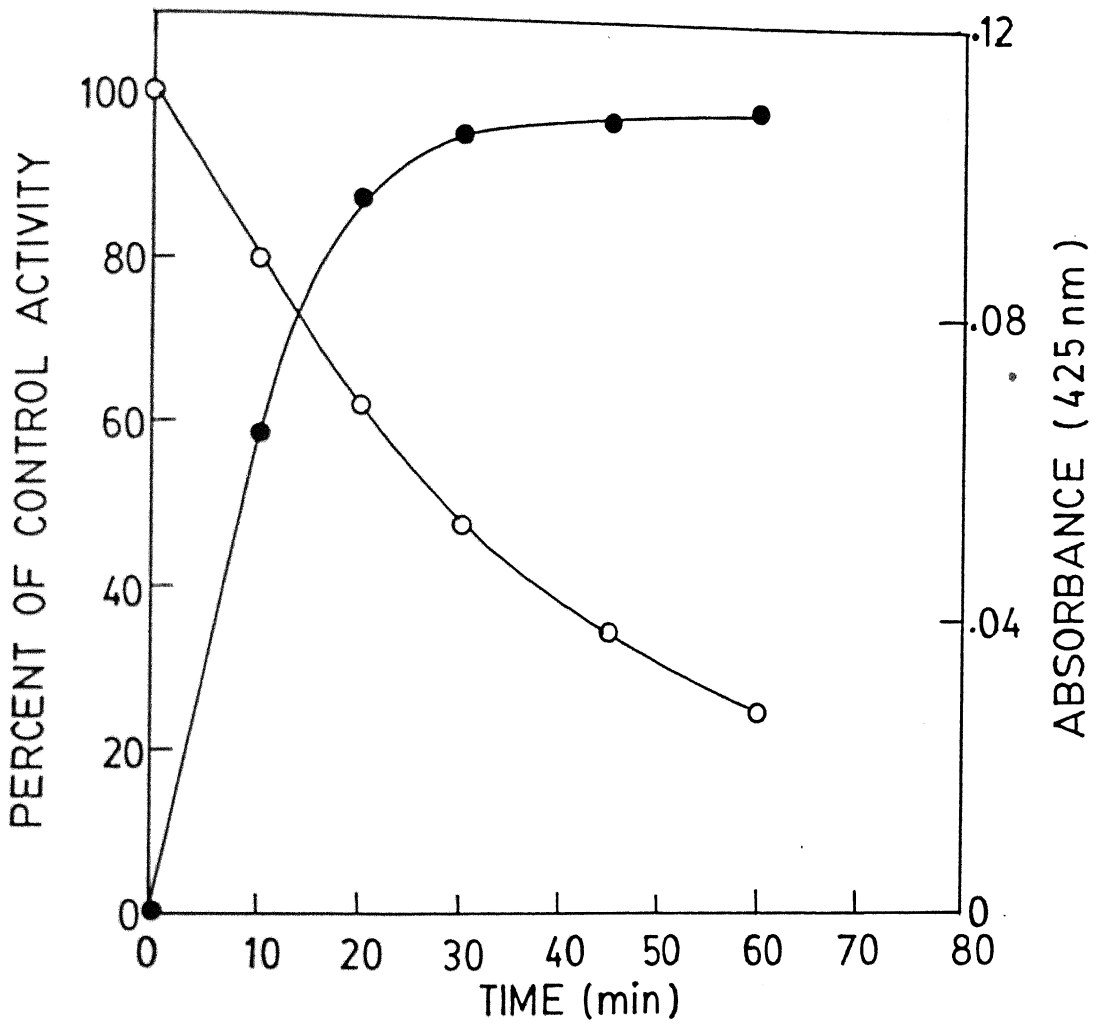


Figure IV.2. Time course of inactivation of dextransucrase (O) and increase in absorbance (●) at 425 nm on treatment with PLP. The enzyme (1.2 mg protein/ml) was incubated with 30 mM PLP in 0.2 M acetate buffer (pH 5.2). In another set enzyme activity was measured as described in "Materials and Methods".

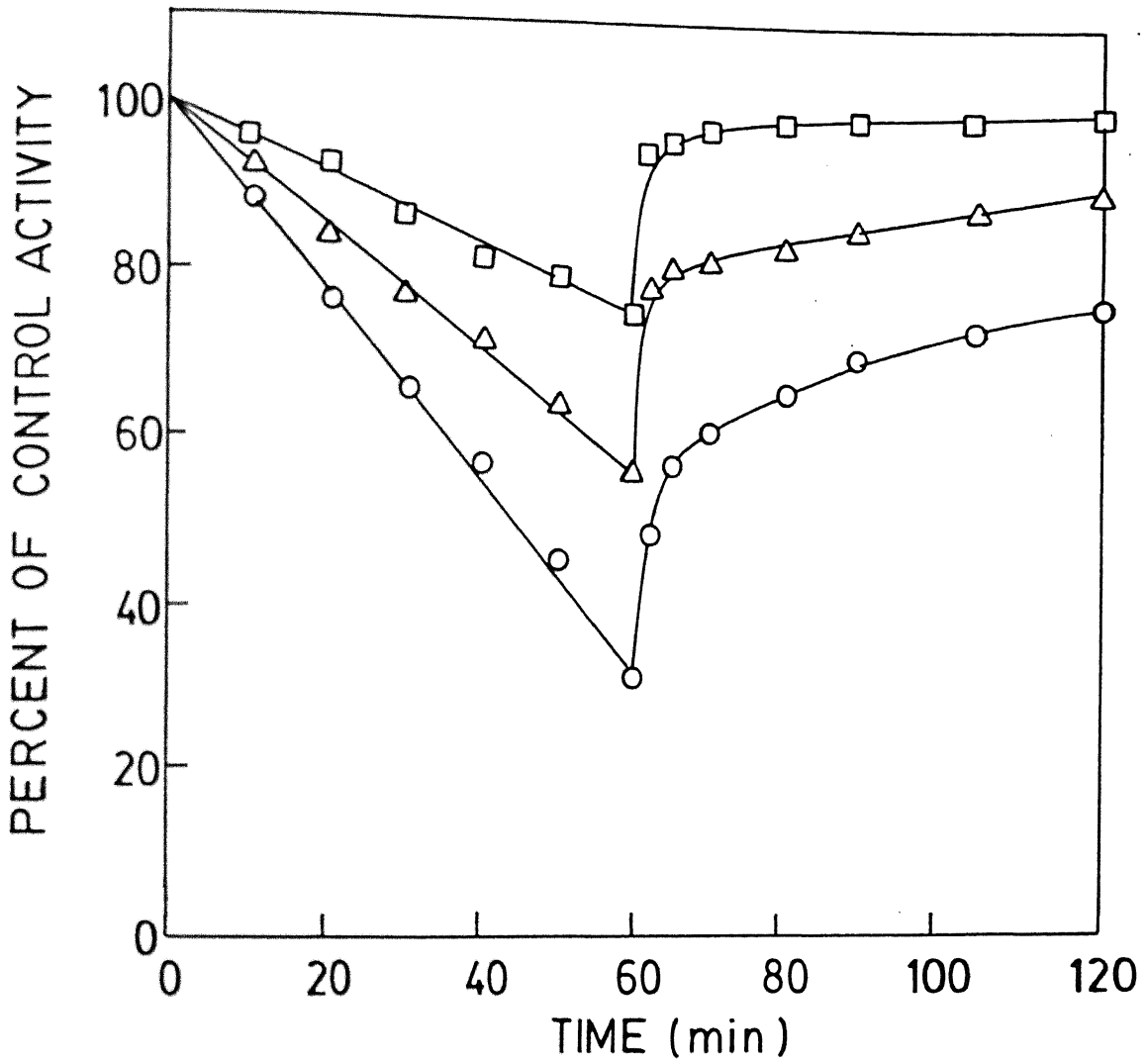


Figure IV.3. Reactivation of dextransucrase by dilution on inactivation by PLP. The enzyme (1.2 mg protein/ml) was incubated with 10 (□), 20 (Δ) and 30 mM (O) PLP for 1h. Reversal of the inactivation was achieved by 20 fold dilution of the reaction mixtures. The enzyme activity was assayed as described in "Materials and Methods".

the inactivation irreversible. These observations suggested the formation of Schiff's base indicating that the inactivation of the enzyme is due to the reaction of PLP with ε-amino group of lysine residue.

IV.3.2 Spectral analysis of pyridoxal 5'-phosphate-modified dextransucrase

The fluorescence emission spectrum of PLP treated dextransucrase followed by sodium borohydride reduction showed a maxima at 397 nm upon excitation at 325 nm and the fluorescence excitation spectrum (λ_{em} = 397 nm) showed a maxima at 325 nm (Fig. IV.4). These results were consistent with the formation of N^{ε}-phosphopyridoxyllysine.

IV.3.3 Effect of substrates on enzyme inactivation by pyridoxal 5'-phosphate

Sucrose rapidly autopolymerizes in the presence of dextransucrase hence the protection experiments only with sucrose were not possible. However, the enzyme is inhibited by EDTA, which could be reversed by the addition of Ca^{2+} ions [1]. The enzyme on incubation with 25 mM and 50 mM EDTA at 30°C resulted in 35% and 60% loss of activity in 30 min (Fig. IV.5). The addition of 25 and 50 mM Ca^{2+} to the EDTA-inactivated

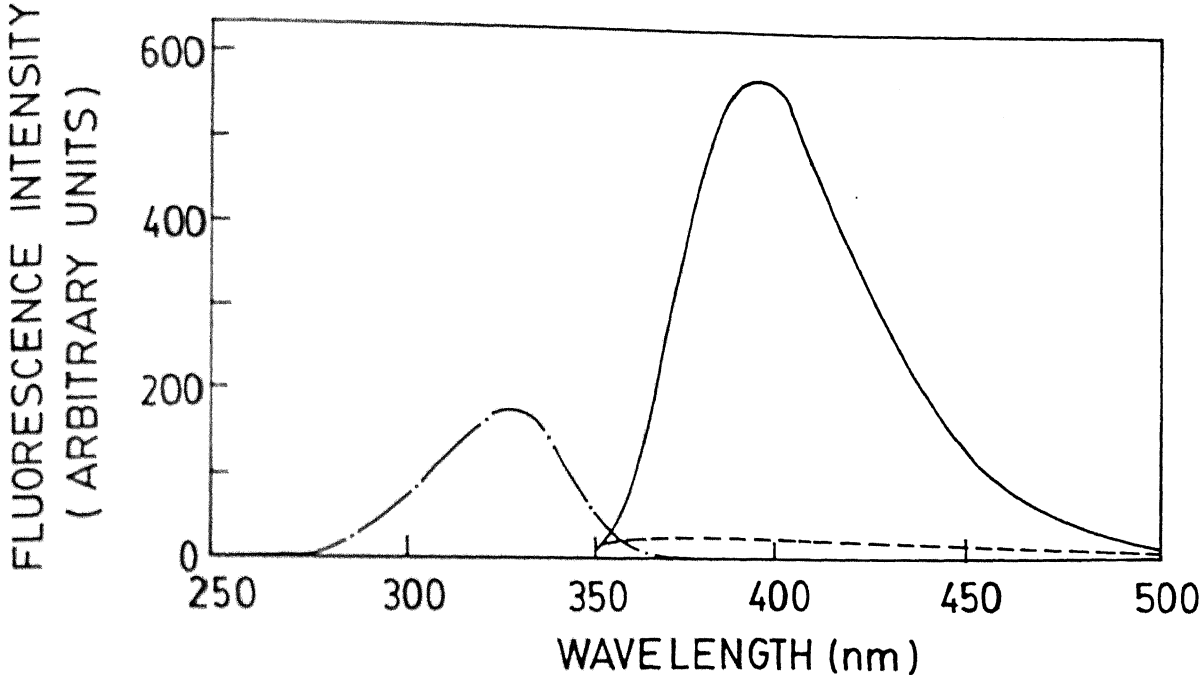


Figure IV.4. Fluorescence excitation and emission spectra of dextransucrase-PLP complex after reduction with sodium borohydride. The enzyme (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 30 mM PLP for 1h at 30°C. The enzyme-inhibitor reaction was terminated by reduction with 50 mM sodium borohydride. The reaction mixture was kept at 0°C for 30 min and then dialyzed extensively against 0.2 M acetate buffer (pH 5.2). The resulting N^{ε}-phosphopyridoxyllysine complex was characterized by fluorescence emission spectrum (———) with excitation wave length at 325 nm and by excitation spectra (— · —) with (λ_{em} = 397 nm). The emission spectra for pure enzyme (-----) with excitation at 325 nm is also shown. For details see "Materials and Methods".

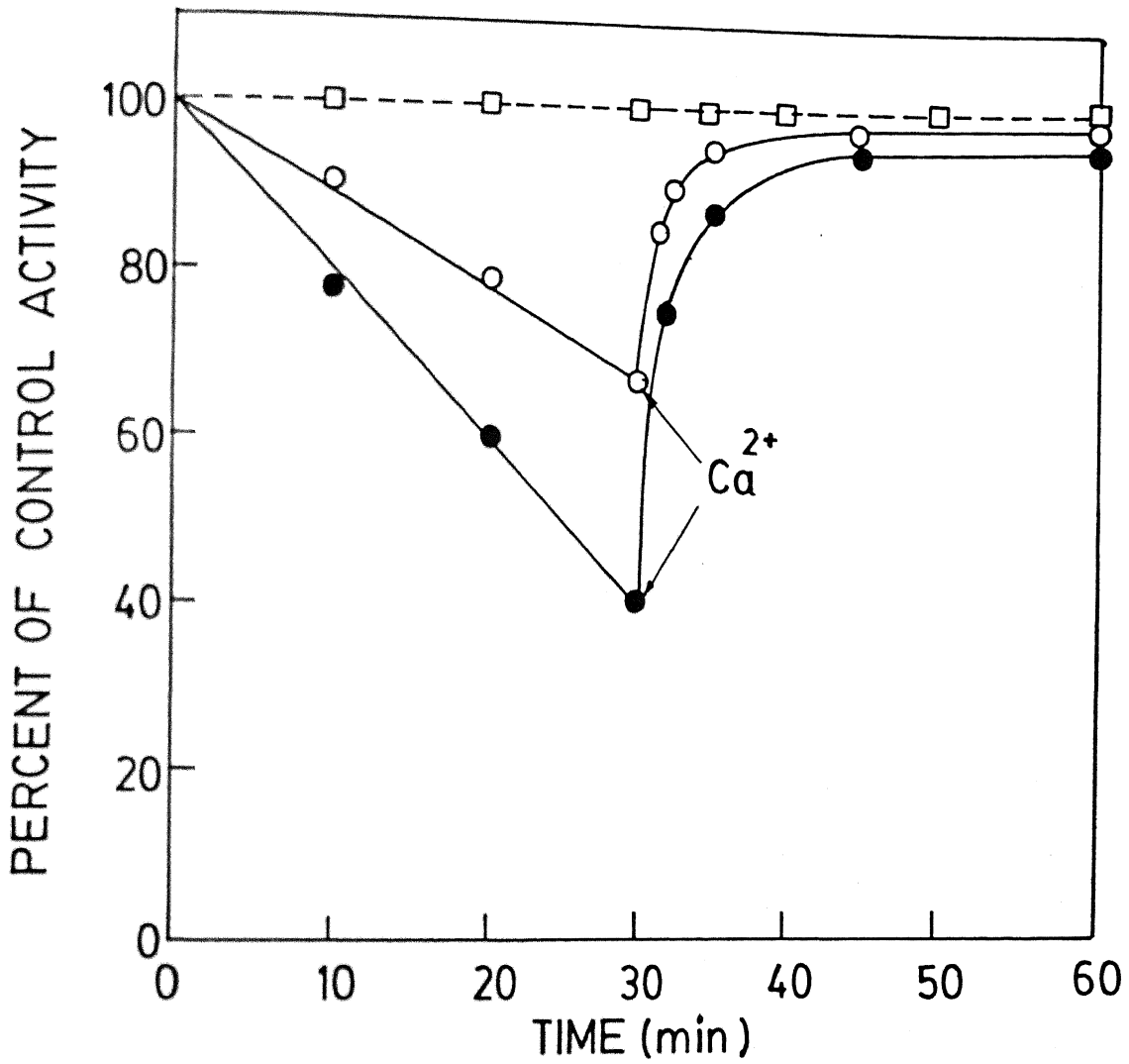


Figure IV.5. Dextransucrase inhibition by EDTA and reactivation by Ca^{2+} ions. The enzyme (1.2 mg protein/ml) was incubated with EDTA 25 mM (○) and 50 mM (●) at 30°C for 30 min followed by the addition of 25 and 50 mM Ca^{2+}, respectively. Aliquots were withdrawn at indicated time intervals and the residual activity was determined as described in "Materials and Methods".

dextransucrase resulted in almost complete reactivation of the enzyme. This approach was used to carry out the protection experiments, in presence of sucrose by quenching the activity of the enzyme and then reactivating it.

D-glucose has been reported to be an acceptor substrate and also used as the active site protecting reagent for dextransucrase [12]. D-glucose is non-reactive (in the absence of sucrose) and meets the structural requirements for binding to the active site of the enzyme. Dextran, the product of the action of dextransucrase can also undergo acceptor reactions to give α-(1⟶3) branched glucosidic linkages, binds to the active site [33]. Thus the effect of sucrose, glucose and dextran on inactivation of PLP was investigated.

The results of the effect of sucrose and acceptor substrates on dextransucrase inactivation by PLP are shown in Table IV.1. A concentration of 300 mM of sucrose provided almost complete protection to the enzyme against inactivation by PLP. Protection of enzyme by sucrose against inactivation was also confirmed by the decrease in fluorescence intensity (Fig. IV.6). Approximately, 88% of the enzyme activity was retained in the presence of 100 mM glucose against PLP inactivation (Table IV.1). In the case of dextran, dextransucrase pre-incubated with dextran lost 40% of its

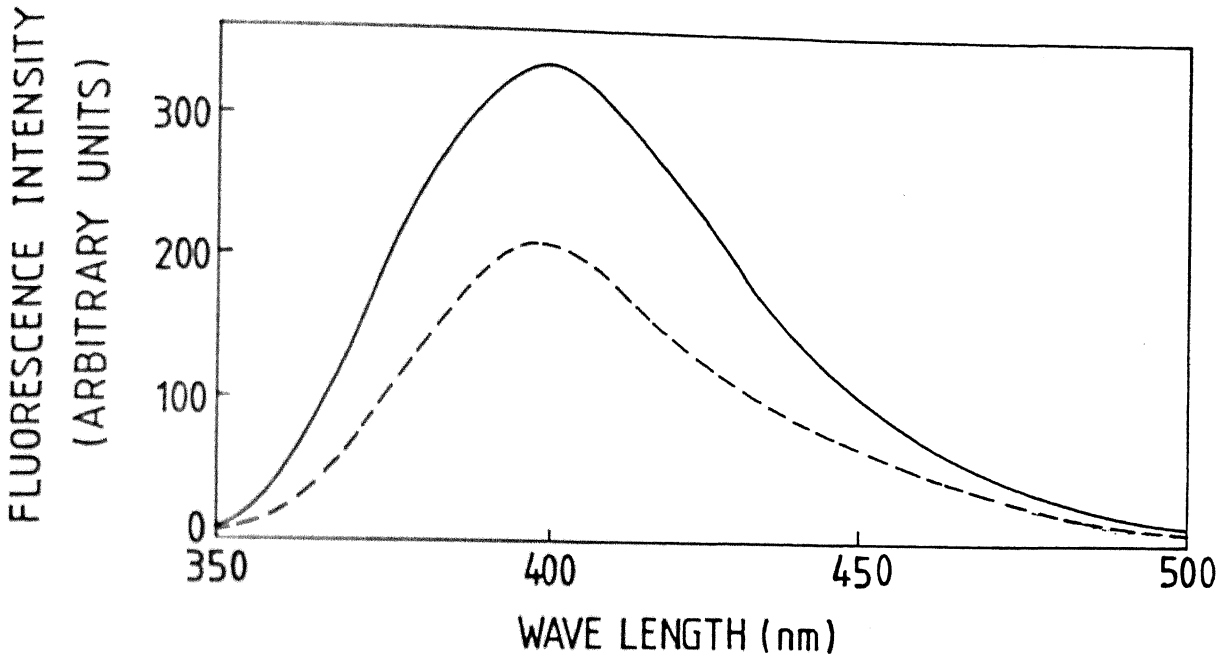


Figure IV.6. Effect of substrate sucrose on the binding of PLP with dextransucrase. The enzyme (1.2 mg protein/ml) was pre-incubated with 50 mM EDTA for 30 min, followed by incubation with 300 mM substrate sucrose for 10 min and finally with 30 mM PLP in 0.2 M acetate buffer (pH 5.2) for 1h 30°C. A control was run concurrently without the sucrose. The fluorescence spectra of control (———) and with 300 mM sucrose (-----) were recorded with excitation wave length 325 nm. For details see "Materials and Methods".

original activity when treated with 30 mM PLP, whereas, in absence of dextran, the enzyme lost 70% of its original activity Table IV.1. This showed that the dextran offered less protection to the enzyme against PLP inactivation. Results of the protection studies indicated that the inactivation of dextransucrase by PLP has resulted from the modification of critical lysine residue(s) present at or near the active site of the enzyme.

Table IV.1

Effect of sucrose and acceptor substrates on inactivation of dextransucrase by PLP. The reagents shown in the table were incubated with dextransucrase (1.2 mg protein/ml) for indicated time period followed by incubation with 30 mM PLP for 1h. Appropriate controls without PLP in each case were run concurrently. For details see Section IV.2.8.

Reagent	Control activity (%)
None	30
EDTA (50 mM, 30 min)	40
Sucrose (100 mM, 10 min)	65
Sucrose (200 mM, 10 min)	80
Sucrose (300 mM, 10 min)	92
Dextran (5 mg/ml, 15 min)	60
Glucose (50 mM, 15 min)	80
Glucose (100 mM, 15 min)	88

IV.3.4 Determination of number of lysine residues involved in reaction of dextransucrase and pyridoxal 5'-phosphate

Stoichiometry of PLP reaction with dextransucrase was determined by the increase in absorbance at 325 nm. The inactivation of dextransucrase by PLP was correlated with the number of modified lysine residues (Fig. IV.7). The plot is non linear, indicating that not all the lysine residues are modified at the same rate. Some lysine residues are more accessible for modification than others. The amino acid analysis of dextransucrase showed that there are 102 lysine residues per enzyme molecule. Although it is known that polar amino acids such as lysyl and arginyl residues are located on the surface in protein molecules, our chemical modification studies showed that out of 102 lysine residues 18 are modified by PLP and most of the other lysyl residues are insensitive to the modification reagent. It is interesting that the modifying reagent appears to react relatively selectively. Since, the modification of enzyme was carried out pH 5.2 it can be expected that only lysine residues having low pK_a can react with the modifying reagent.

The kinetic data were further analyzed using Tsou's statistical method [34] to determine the number of lysine residues essential for enzyme activity. Tsou's statistical

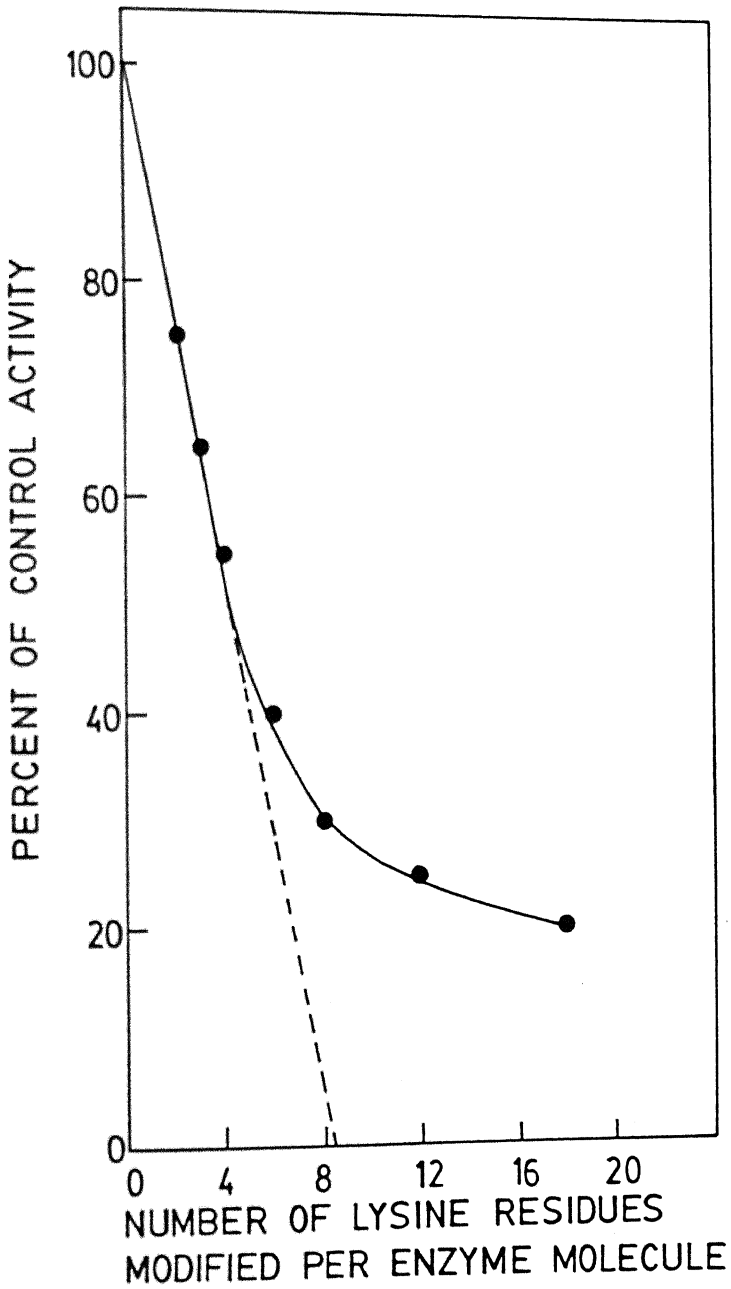


Figure IV.7. The stoichiometry of inactivation of dextransucrase by PLP. The enzyme (1.2 mg protein/ml) was incubated with 30 mM PLP in 0.2 M acetate buffer (pH 5.2). The amount of N^{ϵ}-phosphopyridoxyllysine formed after sodium borohydride reduction, at different time intervals during the enzyme modification was determined by absorbance increase at 325 nm. In a parallel experiment the aliquots were withdrawn from the incubation mixture at same time intervals and quenched with

method has been widely used to determine the number of "essential" amino acid residues modified when more than one residue per enzyme molecule reacts with a modifying/inactivating reagent. This has been used for determining the number of essential arginine residues in transferrins [35], tryptophan synthase [36] and L-threonine dehydrogenase [37], and for histidine in dextransucrase [14] and lysolecithin acyltransferase [38]. Extrapolation of the first phase of the plot to zero enzyme activity showed that 8 lysine residues were modified during this phase (Fig. IV.7). Such an extrapolation does not give the number of lysine residues that are essential for the enzyme activity, but it rather suggests that these 8 lysine residues are modified at a significantly, but not markedly, different rate than are the other lysine residues. The number of essential residues modified under conditions where both essential and nonessential residues are modified at significantly, but not markedly, different rates can be determined by correlating the rate of loss of enzyme activity with the rate of modification of the total number of reactive residues by means of Eq. 1 [34].

$$m = n(1-x) = n - p(A/A_0)^{1/1} - (n-p)(A/A_0)^{\alpha/1} \qquad (1)$$

Where m is the number of lysine residue modified, n is the total number of lysine residues modified to give complete inactivation of enzyme, p is the total number of lysine residues including i essential residues modified at the rate constant K_1, $n-p$ is the number of lysine residues modified at rate constant $K_2 (K_2 = \alpha K_1)$, A/A_0 is the fraction of enzyme activity at any time during the modification and x is the total fraction of unmodified residues remaining at any value of A/A_0. Eq. 1 can be rearranged to give Eq. 2.

$$\log[nx/(A/A_0)^{1/i} - p] = (\alpha-1)/i \log(A/A_0) + \log(n-p) \qquad (2)$$

$\log[nx/(A/A_0) - p]$ was plotted against $\log(A/A_0)$ using the values of $n = 18$, $p = 8$, and $i = 1,2,3,4$. When $i = 1$, the best linear fit to the equation, with linear regression variance $R^2 = 4.57 \times 10^{-4}$, was obtained. The results suggested that only one lysine residue per enzyme molecule is essential for the activity.

IV.3.5 Inactivation of dextransucrase by 2,4,6-trinitrobenzene-sulphonic acid

The inactivation of dextransucrase by TNBS was dependent on the inhibitor concentration as well the time of incubation. The treatment of dextransucrase with TNBS in the concentration range 10-30 mM led to the loss of enzyme activity and 85% inactivation occurred in 1h at 30 mM TNBS (Fig. IV.8). Plots of log (residual activity) *versus* time of incubation in the concentration range 10-30 mM of the inhibitor were linear, indicating that the inactivation followed pseudo-first order kinetics (Fig. IV.8). A plot of the observed pseudo-first order rate constants against the concentration of TNBS gave a straight line passing through the origin (Fig. IV.8, inset), from which a second order rate constant of 2.66 $M^{-1}min^{-1}$ was obtained. The inactivation pattern corresponded with the increase of absorbance at 367 nm (Fig. IV.9) and there was no increase in absorbance after 60 min of TNBS incubation with the enzyme which resulted in 85% activity loss. The inactivation of dextransucrase by TNBS was found irreversible as the dilution and dialysis of the incubation mixture could not reverse the activity loss. These observations suggested the formation of trinitrophenyl derivative by the reaction of TNBS with ε-amino group of lysine residue.

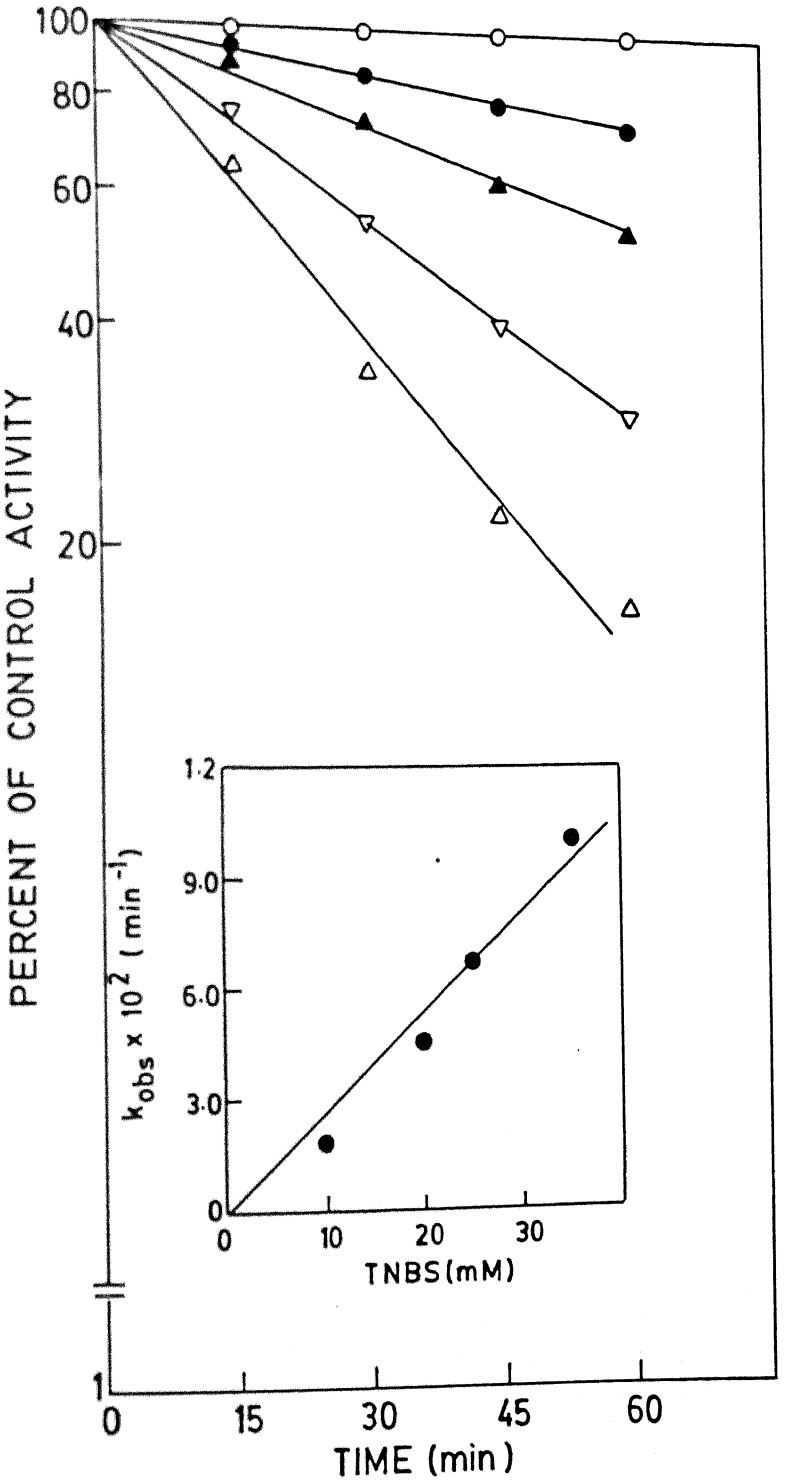


Figure IV.8. Kinetics of inactivation of dextransucrase by TNBS. The enzyme (1.2 mg protein/ml, 30 U/mg) in 0.2 M acetate buffer (pH 5.2), was incubated with 0 (O); 10 (●); 20 (▲); 25 (▽); and 30 mM (Δ) TNBS at 30°C. Aliquots were withdrawn at indicated time intervals and the residual activity was determined as described in "Materials and Methods". Inset: a plot of observed pseudo-first order rate constant *vs* TNBS concentrations, from which a second order rate constant was obtained.

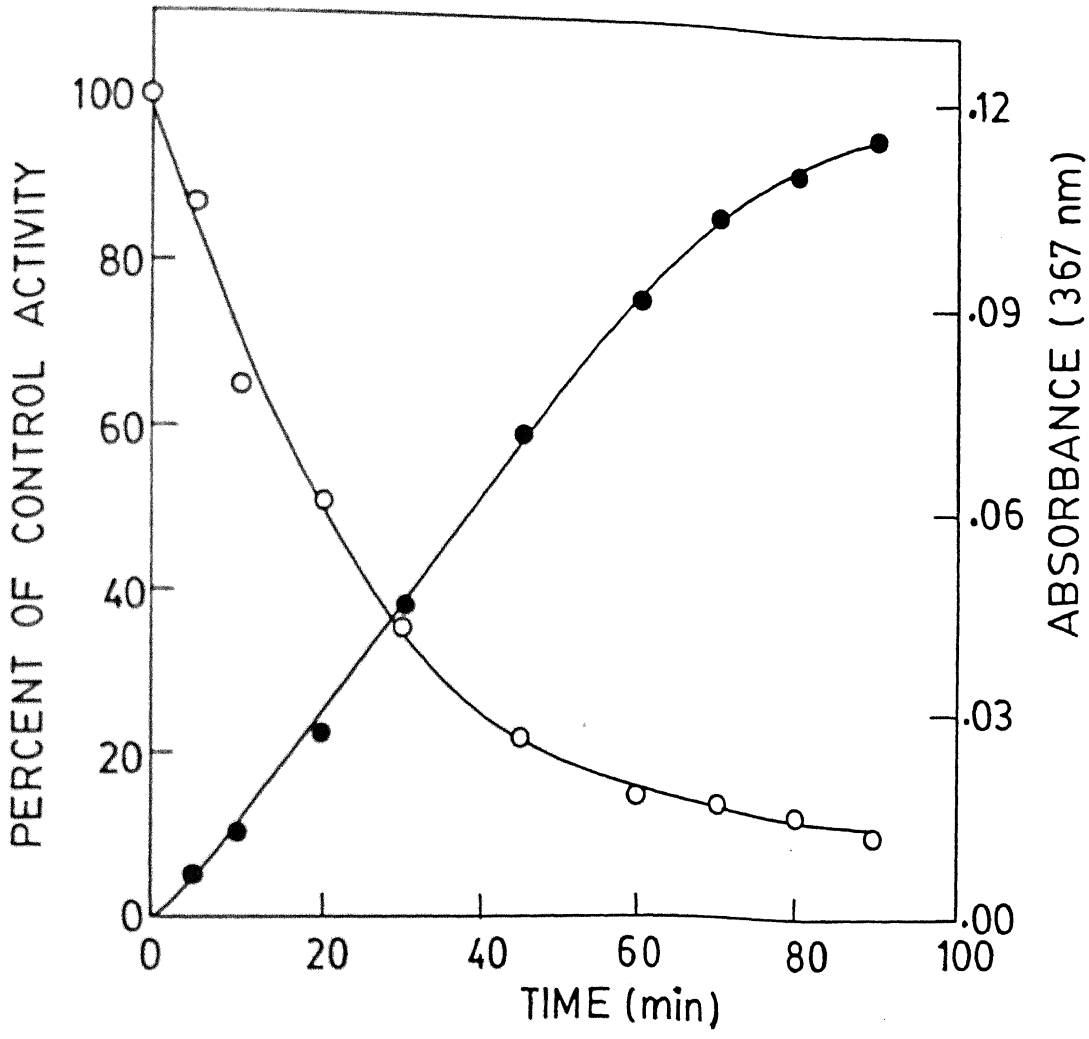


Figure IV.9. Time course of inactivation of dextransucrase (O) and increase in absorbance (●) at 367 nm on treatment with TNBS. Dextransucrase (1.2 mg/ml) was incubated with 30 mM TNBS in 0.2 M acetate buffer (pH 5.2). In another set enzyme activity was measured as described in "Materials and Methods".

IV.3.6 Stoichiometry of inactivation by 2,4,6-trinitrobenzene-sulphonic acid

Stoichiometry of TNBS reaction with dextransucrase was determined by monitoring the absorbance enhancement recorded at 367 nm [22,29]. The percentage of residual dextransucrase activity was plotted as a function of amount of TNBS incorporated per mol of the enzyme (Fig. IV.10). Approximately, 4 mol of trinitrophenyl derivative of ε-amino group of lysine were formed per mol of enzyme. However, when the data were extrapolated to zero enzyme activity, approximately 1 mol of TNBS was incorporated per mol of dextransucrase.

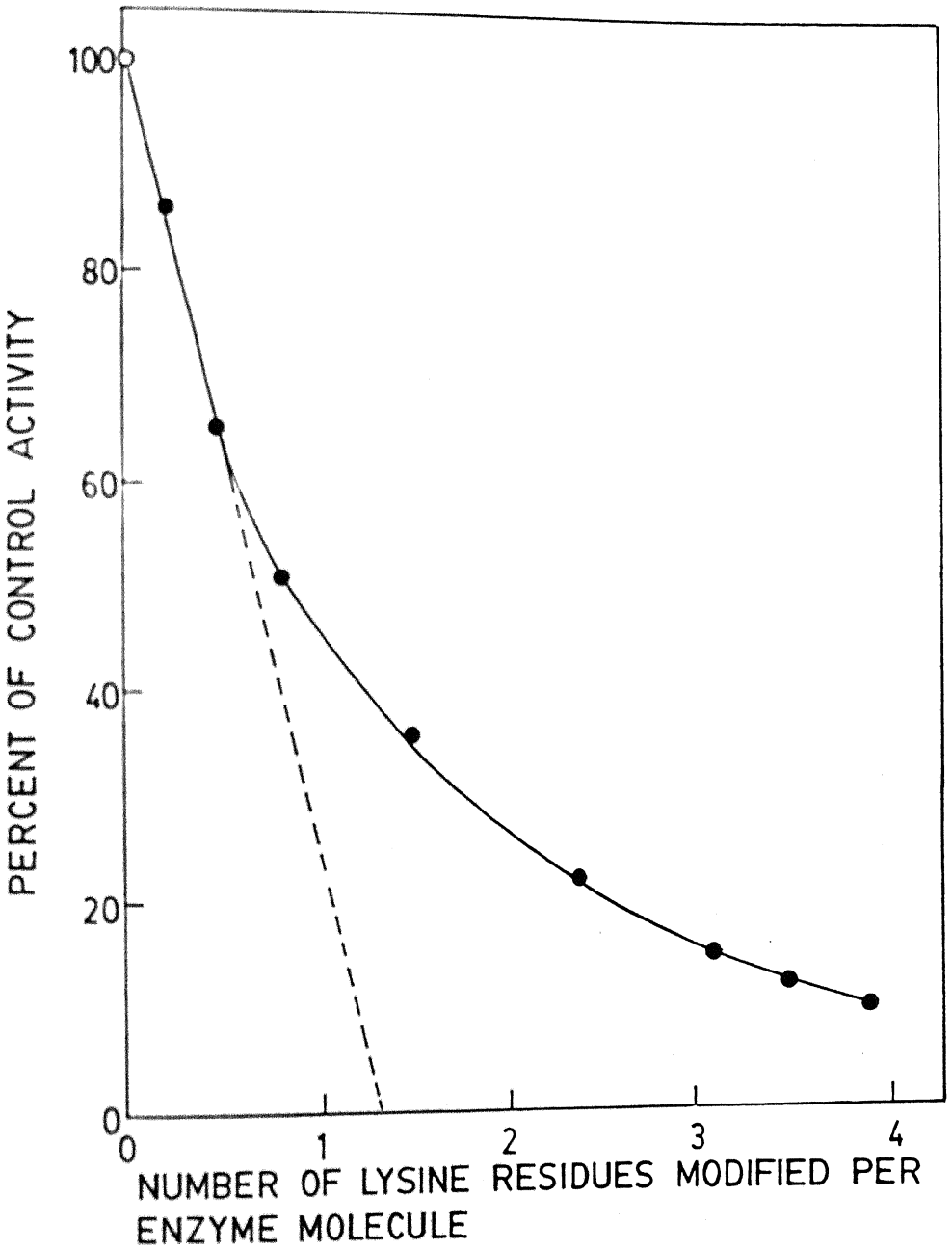


Figure IV.10. Stoichiometry of inactivation of dextransucrase by TNBS. The enzyme (1.2 mg protein/ml) was incubated with 30 mM TNBS in 0.2 M acetate buffer (pH 5.2). The amount of trinitrophenyl derivative of ε-amino group of lysine residue formed at different time intervals during the enzyme inactivation was determined by absorbance increase at 367 nm. In a parallel experiment the aliquots were withdrawn from the incubation mixture at same time intervals the residual enzyme activity was determined as described in "Materials and Methods".

IV.4 SUMMARY

The inactivation of dextransucrase by PLP and TNBS was concentration and time dependent. The reaction by both the inhibitors followed pseudo-first order kinetics, which indicated a direct correlation between the inactivation and the modification of dextransucrase. The inactivation of enzyme by PLP was reversible as it could be reversed completely by dilution or dialysis, but TNBS caused irreversible inactivation. These results showed that the inactivation of dextransucrase by PLP and TNBS are due to the specific modification of ε-amino group of lysine and not due to non-covalent binding of the inhibitor causing enzyme denaturation.

The characteristic increase in absorbance at 325 nm in the case of PLP enabled the correlation of the degree of inactivation to the number of lysine residues modified. There are 102 lysine residues per enzyme molecule and out of these, 18 were modified by PLP. The treatment of the kinetic data of inactivation using Tsou's statistical method [34] suggested the involvement of one lysine residue in the PLP-dextransucrase reaction. Similarly, the stoichiometry of the inactivation of dextransucrase by TNBS also showed the modification of one mol of lysine per mol of enzyme. Substrate, sucrose or the acceptor

substrate, glucose protected the enzyme against PLP inactivation. These results proved the presence of one essential lysine residue at the active site.

It has been reported earlier that the two identical sucrose sites and one acceptor binding site constitute the active site of dextransucrase [15]. Presence of two aspartate [13] and two histidine residues [14] at the active site have been reported. Our results have conclusively shown that one lysine residue is required for activity of dextransucrase. Although the exact role of lysine in catalysis is not known but it is possible that this lysine is present between the two identical sucrose binding sites or at the acceptor binding site and is essential for maintaining the conformation of active site. The modification of this lysine residue by the inhibitors changes the conformation of the active site leading to the enzyme inactivation.

The present investigations have convincingly demonstrated the presence of an essential lysine residue at the active site of dextransucrase. Further insight of the role of essential lysine residue in the reaction mechanism of dextransucrase may be provided by the mapping the reactive peptide of the active site and altering the catalytic residue by site-directed mutagenesis.

REFERENCES

1. Robyt, J.F. and Walseth, T.F. (1979) Carbohydr. Res. 68, 95-111.

2. Kobayashi, M. and Matsuda, K. (1980) Biochim. Biophys. Acta 614, 46-62.

3. Kobayashi, M. and Matsuda, K. (1986) J. Biochem. 100, 615-621.

4. Miller, A.W., Eklund, S.H. and Robyt, J.F. (1986) Carbohydr. Res. 147, 119-133.

5. Willemot, R.M., Monsan, P. and Durand, G. (1988) Ann. N. Y. Acad. Sci. 542, 169-172.

6. Fu, D. and Robyt, J.F. (1990) Prep. Biochem. 20, 93-106.

7. Goyal, A. and Katiyar, S.S. (1994). J. Microbiol. Methods 20, 225-231.

8. Neely, W.B. (1959) Arch. Biochem. Biophys., 79, 297-301.

9. Kobayashi, M., Yokoyama, I. and Matsuda, K. (1986) Agric. Biol. Chem., 50, 2585-2590.

10. Robyt, J.F., Kimble, B.K. and Walseth, T.F. (1974) Arch. Biochem. Biophys., 165, 634-640.

11. Huang, S., Lee, C. and Mayer, R.M. (1979) Carbohydr. Res. 74, 287-300.

12. Kobs S.F. and Mayer R.M. (1991) Carbohydr. Res. 211, 317-326.

13. Mooser, G., Hefta, S.A., Paxton, R.J., Shively, J.E. and Lee, T.D. (1991) J. Biol. Chem., 266, 8916-8922.

14. Fu, D. and Robyt, J.F. (1988) Carbohydr. Res., 183, 97-109.

15. Su, D. and Robyt, J.F. (1994) Arch. Biochem. Biosphys., 308, 471-476.

16. Talbot, J.-C., Gros, C., Cosson, M.-P. and Pantaloni, D. (1977) Biochim. Biophys. Acta 494, 19-32.

17. Katiyar, S.S. and Porter, J.W. (1982) Biochem. Biophys. Res. Commun. 107, 1219-1223.

18. Shourky, S., Stoops, J.K., Wakil, S. J. (1983) Arch. Biochem. Biophys. 226, 224-230.

19. Dong, Q. and Fromm, H.J. (1990) J. Biol. Chem. 265, 6235-6240.

20. Green, P.C., Latshaw, S.P., Ladror, U.S. and Kemp, R.G. (1992) Biochemistry, 31, 4815-4821.

21. Goyal, A. and Katiyar, S.S. (1994) J. Enz. Inhibition (in press).

22. Freedman R.B. and Radda, G.K. (1969) Biochem. J., 114, 611-619.

23. Carlberg, I. and Mannerwick, B. (1979) FEBS Lett. 98, 263-266.

24. Nelson, N., (1944) J. Biol. Chem., 153, 375-380.

25. Somogyi, M. (1945) J. Biol. Chem., 160, 69-73.

26. Lilley, K.S. and Engel, P.C. (1992) Eur. J. Biochem. 207, 533-540.

27. Robyt, J.F. and Walseth, T.F. (1978) Carbohydr. Res., 61, 433-445.

28. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

29. Bell, J.E. and Bell, E.T. Proteins and Enzymes, (1988) p-152, Prentice-Hall, New Jersey.

30. Salnikow, J., Liao, T.-H., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 1480-1488.

31. Chen, S. and Engel, P.C. (1975) Biochem. J. 149, 627-635.

32. Thaniyavaran, S., Taylor, K.G., Singh, S. and Doyle, R.J. (1982) Infect. Immun. 37, 1101-1111.

33. Robyt, J.F. and Taniguchi, H. (1976) Arch. Biochem. Biophys., 174, 129-135.

34. Tsou, C.L. (1962) Sci. Sin. (*Engl. Edn.*), 11, 1535-1558.

35. Rogers, T.B., Borresen, T. and Feeney, R.E. (1978) Biochem. 17, 1105-1109.

36. Eun, H.M. and Miles, E.W. (1984) Biochem. 23, 6484-6491.

37. Epperly, B.R. Dekker, E.E. (1989) J. Biol. Chem. 264, 18296-18301.

38. Perez-Gil, J., Martin, J., Acebal, C. and Arche, R. (1889) Arch. Biochem. Biophys. 269, 562-568.

26. Lilley, K.S. and Engel, P.C. (1992) Eur. J. Biochem. 207, 533-540.

27. Robyt, J.F. and Walseth, T.F. (1978) Carbohydr. Res., 61, 433-445.

28. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

29. Bell, J.E. and Bell, E.T. Proteins and Enzymes, (1988) p-152, Prentice-Hall, New Jersey.

30. Salnikow, J., Liao, T.-H., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 1480-1488.

31. Chen, S. and Engel, P.C. (1975) Biochem. J. 149, 627-635.

32. Thaniyavaran, S., Taylor, K.G., Singh, S. and Doyle, R.J. (1982) Infect. Immun. 37, 1101-1111.

33. Robyt, J.F. and Taniguchi, H. (1976) Arch. Biochem. Biophys., 174, 129-135.

34. Tsou, C.L. (1962) Sci. Sin. (*Engl. Edn.*), 11, 1535-1558.

35. Rogers, T.B., Borresen, T. and Feeney, R.E. (1978) Biochem. 17, 1105-1109.

36. Eun, H.M. and Miles, E.W. (1984) Biochem. 23, 6484-6491.

37. Epperly, B.R. Dekker, E.E. (1989) J. Biol. Chem. 264, 18296-18301.

38. Perez-Gil, J., Martin, J., Acebal, C. and Arche, R. (1889) Arch. Biochem. Biophys. 269, 562-568.

CHAPTER V

ACTIVE SITE MAPPING STUDIES ON DEXTRANSUCRASE USING *o*-PHTHALALDEHYDE

V.1 INTRODUCTION

Dextransucrase {Sucrose: 1,6-α-D glucan 6-α-D glucosyl transferase, EC 2.4.1.5} from *Leuconostoc mesenteroides* NRRL B-512F exists in either single or multiple forms [1-5]. Extensive investigations have been carried out regarding the structural organization of the active site of the enzyme [6-8]. Separate sucrose and dextran binding domains have been identified in *Streptococcus mutans* dextransucrase [9]. Sucrose-induced conformational changes to align domains into a functional active site have been suggested as the reaction is essentially irreversible [9]. It has been proposed that *Leuconostoc mesenteroides* NRRL B-512F dextransucrase has two sucrose binding sites and one acceptor binding site at the active site [10]. There are few reports on specific amino acid residues present at the active site. Two pertinent amino acid functional groups have been implicated in mechanism of glucosyl

transfer from sucrose. An aspartate containing active site peptide bound covalently to glucosyl group was isolated from dextransucrases of *Streptococcus sobrinus* [11]. By chemical modification studies using diethyl pyrocarbonate it was shown that the two essential histidine residues are present at the active site [12]. A nucleophilic carboxylate group of aspartic acid stabilizes the carbonium ion in equilibrium with the covalent glucosyl-enzyme complex [11]. The imidazolium groups of histidine residues donate protons to the leaving D-fructosyl moieties and the resulting imidazole groups facilitate the formation of α-(1⟶6)-glucosidic linkage [12].

Chemical modification studies have been carried out using fluorogenic bifunctional reagent, *o*-phthalaldehyde with various enzyme systems [13-27]. Lysine and cysteine have been identified as active site residues by the use of *o*-phthalaldehyde in various enzymes [14-17,20,22-26]. This reagent specifically binds to the sulfhydryl group of cysteine and ε-amino group of lysine and gives a fluorescent, isoindole derivative. Formation of isoindole derivative is possible only when these two functional groups are in close proximity to each other. Thus, it provides vital information about the distance and orientation of these residues at the active site of enzyme. Present investigations reveal the existence of a critical lysine residue at the active site of dextransucrase, based on the chemical modification studies by *o*-phthalaldehyde [27].

V.2. MATERIALS AND METHODS

Materials

Pyridoxal 5′-phosphate (PLP), N-ethyl maleimide (NEM), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), *o*-phthalaldehyde, L-cysteine and β-mercaptoethanol and were obtained from Sigma Chemical Company, USA. All other chemicals used were of highest purity grade, commercially available.

Methods

V.2.1 Enzyme preparation and activity assay

Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F purified by fractionation with polyethylene glycol 400 as reported previously [5], had a specific activity of 30 U/mg protein. One unit of dextransucrase activity is defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute at 30°C and pH 5.2. For all modification reactions dextran free enzyme was used and the native dextran present in the enzyme was removed by dextranase treatment [2].

The enzyme activity was determined by measuring the rate of production of reducing sugar. The activity was assayed at 30°C in 0.2 M sodium acetate buffer (pH 5.2). The assay mixture (1.0 ml) containing 10% substrate sucrose in 0.2 M acetate buffer (pH 5.2) and the enzyme solution was incubated at 30°C for 20 min. Aliquots (0.1-0.2 ml), from the reaction mixture were analyzed for reducing sugar by the method of Nelson [28]

and Somogyi [29]. The analytical procedures are described in Chapter IV, section IV.II.1.

V.2.2 Enzyme inactivation by o-phthalaldehyde

The solution of *o*-phthalaldehyde was prepared in 0.2 M acetate buffer (pH 5.2) containing 1% distilled methanol. Controls containing same amount of methanol did not show any effect on the enzyme activity. The enzyme (1.2 mg protein/ml) was incubated with the indicated concentrations of *o*-phthalaldehyde at 30°C. At different time intervals 200 µl aliquots were withdrawn from a total of 2.0 ml incubation mixture and added to 0.5 ml solution containing 150 µl each of 20 mM cysteine and 5 mM β-mercaptoethanol. This terminated the further reaction of *o*-phthalaldehyde with the enzyme. To above mixture 0.5 ml of 20% sucrose was added and incubated for 20 min. The residual enzyme activity was determined by taking aliquots (0.1-0.2 ml) from the assay mixture (1.0 ml) and analyzed for reducing sugar as described earlier. Control mixtures without *o*-phthalaldehyde were run concurrently.

V.2.3 Spectral studies on o-phthalaldehyde binding with dextransucrase

Dextransucrase (1.2 mg/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 5 mM *o*-phthalaldehyde at 30°C for 30 min. Fluorescence emission spectra of isoindole derivative

resulting from dextransucrase-*o*-phthalaldehyde reaction were recorded by excitation at wave length 337 nm on luminescence spectrometer, Perkin Elmer (Model, LS 50B). Fluorescence intensity enhancement due to isoindole derivative formation was measured at 417 nm. The fluorescence was recorded in cuvettes of 1 cm light path at 30°C.

V.2.4 Effect of preincubation of dextransucrase with β-mercaptoethanol on inactivation by o-phthalaldehyde

Dextransucrase (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) reacted with 5 mM *o*-phthalaldehyde at 30°C in absence and presence of 5 mM β-mercaptoethanol. The reactions were monitored continuously by recording the increase in absorbance at 337 nm and assaying for enzyme activity as described earlier. Absorbance spectra and fluorescence emission spectra of isoindole derivatives were recorded on UV-Vis spectrophotometer Shimadzu (Model UV-160A) and luminescence spectrometer, Perkin Elmer (Model, LS 50B)., respectively. The absorbance and fluorescence were recorded in cuvettes of 1 cm light path at 30°C.

V.2.5 Effect of substrates on inactivation of dextransucrase by o-phthalaldehyde

For protection experiments, the enzyme (1.2 mg protein/ml, 30 U/mg) was incubated with 50 mM EDTA in 0.2 M acetate buffer (pH 5.2) at 30°C for 30 min. The sucrose, was then added and incubated for another 15 min at 30°C prior to the addition of 5 mM *o*-phthalaldehyde. After 30 min the residual activity was determined following the same procedure as described in Chapter IV, Section IV.2.2. The assay mixture contained 50 mM of Ca^{2+} for reactivation of the enzyme. The enzyme preincubated with EDTA followed by treatment with Ca^{2+} without PLP was used as control.

The enzyme (1.2 mg protein/ml) was incubated with 50 mM and 100 mM glucose for 15 min at 30°C prior to the addition of 5 mM *o*-phthalaldehyde. After 30 min the residual activity was determined following the procedure described in Section IV.2.2. The appropriate controls without *o*-phthalaldehyde and with glucose were run concurrently.

V.2.6 Stoichiometry of the reaction of dextransucrase with o-phthalaldehyde

The stoichiometry of the reaction was determined by incubating the enzyme (1.2 mg/ml) in 0.2 M acetate buffer (pH 5.2) with 5 mM *o*-phthalaldehyde for 30 min at 30°C. The number of mol of *o*-phthalaldehyde incorporated per mol of enzyme was

determined by taking the ratio of concentration of isoindole derivative using the absorbance coefficient of 7660 $M^{-1}cm^{-1}$ at 337 nm [23] and concentration of enzyme by Lowry *et al.* method [30]. The molecular weight of dextransucrase was taken as 188,000 [5].

V.2.7 Effect of DTNB and PLP preincubation of dextransucrase on o-phthalaldehyde binding

The enzyme (1.2 mg/ml) was incubated with 5 mM DTNB for 15 min prior to incubation with 5 mM *o*-phthalaldehyde at 30°C. The incubation of enzyme with two inhibitors was characterized by monitoring the fluorescence emission spectra before and after incubation with *o*-phthalaldehyde.

The enzyme (1.2 mg/ml) was first incubated with 30 mM pyridoxal 5′-phosphate (PLP) at 30°C for 1h followed by incubation with 5 mM *o*-phthalaldehyde for 30 min. The fluorescence emission spectra were monitored before and after the dialysis of above incubation mixture.

V.2.8 Effect of dextransucrase denaturation on o-phthalaldehyde reaction

The enzyme (1.2 mg/ml) was incubated with 8 M urea or kept in boiling water for 2 min and incubated with 5 mM *o*-phthalaldehyde in acetate buffer (pH 5.2) for 30 min at 30°C. A control was run in parallel and fluorescence spectra were

recorded after 30 min of incubation. The fluorescence spectra were recorded with excitation wave length 337 nm.

V.2.9 Determination of number of sulfhydryl groups

The number of total sulfhydryl groups per molecule of dextransucrase were determined using DTNB by the method as described by Ellman [31].

V.2.10 Amino acid composition of dextransucrase

The amino acid composition of dextransucrase was determined by hydrolyzing the lyophilized enzyme (10 mg protein) with 6 N HCl under nitrogen in sealed tubes at 110°C for 24 h and analyzed by amino acid analyzer (Model, LKB 4101, Sweden) according to the procedure of Salnikow *et al.* [32]. A spectrophotometric estimation of tryptophan was made by the method of Goodwin and Morton [33]. The details of amino acid analysis are described in Chapter III, Section III.2.8.

V.3 RESULTS AND DISCUSSION

V.3.1 Kinetics of o-phthalaldehyde-inactivated dextransucrase

Dextransucrase was rapidly and irreversibly inactivated by *o*-phthalaldehyde (Fig. V.1). The inactivation was dependent on concentration of *o*-phthalaldehyde and the time of incubation. The residual enzyme activity was plotted on semi-logarithmic scale with the time of incubation. The linear relationships

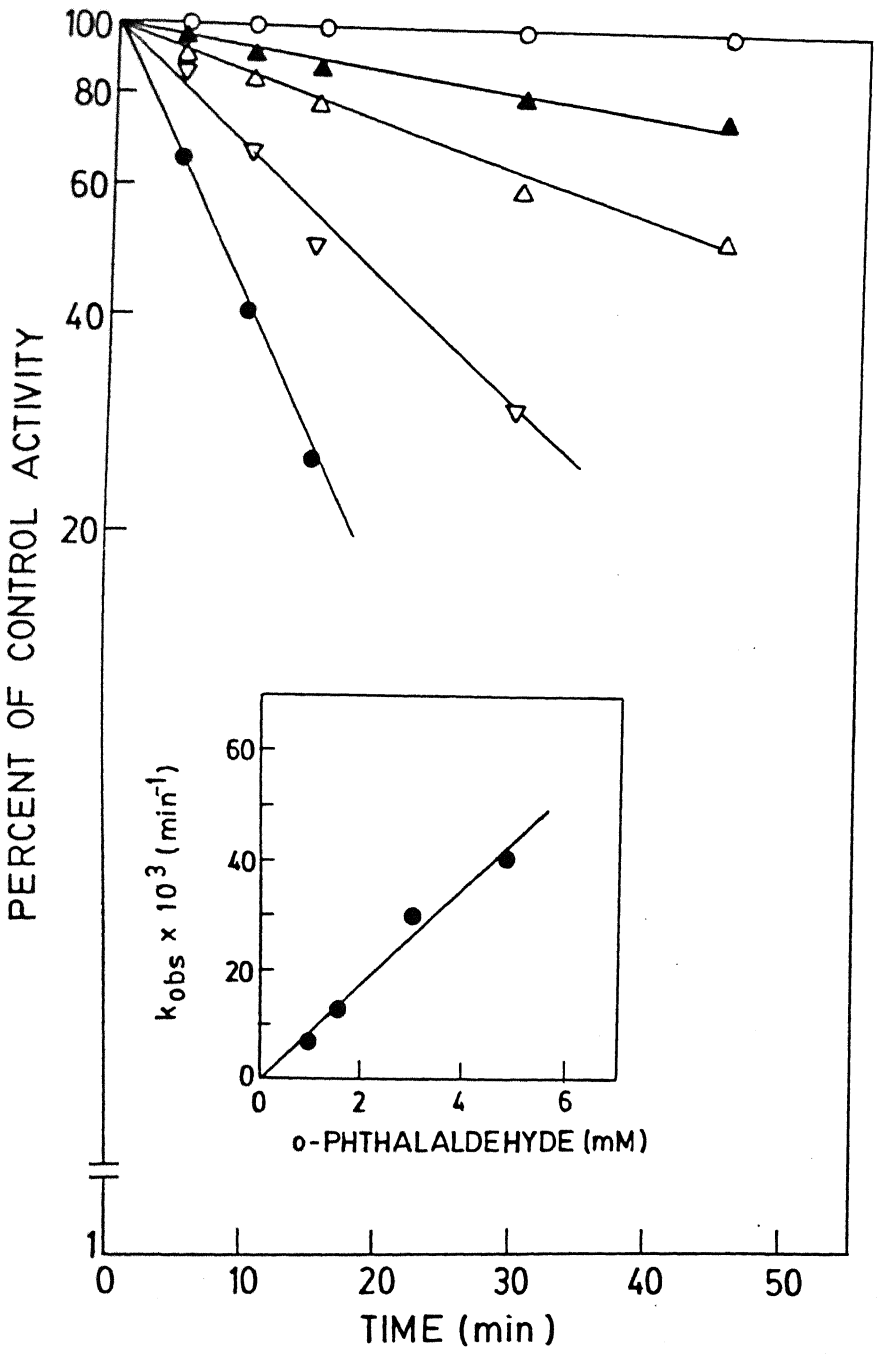


Figure V.1. Kinetics of inactivation of dextransucrase by *o*-phthalaldehyde. The enzyme (1.2 mg/ml, 30 U/mg protein) in 0.2 M acetate buffer (pH 5.2), was incubated with 0 (O); 1 (▲); 1.5 (Δ); 3 (∇); and 5 mM (●) *o*-phthalaldehyde at 30°C. Aliquots were withdrawn at indicated time intervals and the residual activity was determined as described in "Materials and Methods". Inset: a plot of observed pseudo-first order rate-constant *vs* initial *o*-phthalaldehyde concentrations, from which a second-order rate-constant was obtained.

obtained during the initial phase of activity loss indicated that the inactivation followed pseudo-first order kinetics (Fig. V.1). The second order rate constant of 8.75 $M^{-1}min^{-1}$ was obtained by plotting the observed pseudo- first order rate constants against the concentrations of *o*-phthalaldehyde (Fig. V.1, Inset). The modification of dextransucrase with *o*-phthalaldehyde led to an increase in fluorescence intensity at 417 nm on excitation at wave length 337 nm, which was proportional to the degree of inactivation (Fig. V.2). The inactivation of enzyme by *o*-phthalaldehyde was irreversible as prolonged dialysis of the incubation mixture could not reverse the loss of activity.

V.3.2 Characterization of isoindole formation due to dextransucrase-o-phthalaldehyde reaction

The fluorescence emission spectrum of dextransucrase-*o*-phthalaldehyde adduct showed a characteristic fluorescence maxima at 417 nm when excited at 337 nm (Fig. V.3). The fluorescence intensity was not changed even when recorded after 24 h showing that the adduct was stable. These results were consistent with the isoindole ring formation in which the sulfhydryl group of cysteine and ε-amino group of lysine are involved in the binding of *o*-phthalaldehyde. Furthermore, the isoindole formation can occur only when these two functional groups are not more than 3 A° far from each other [23].

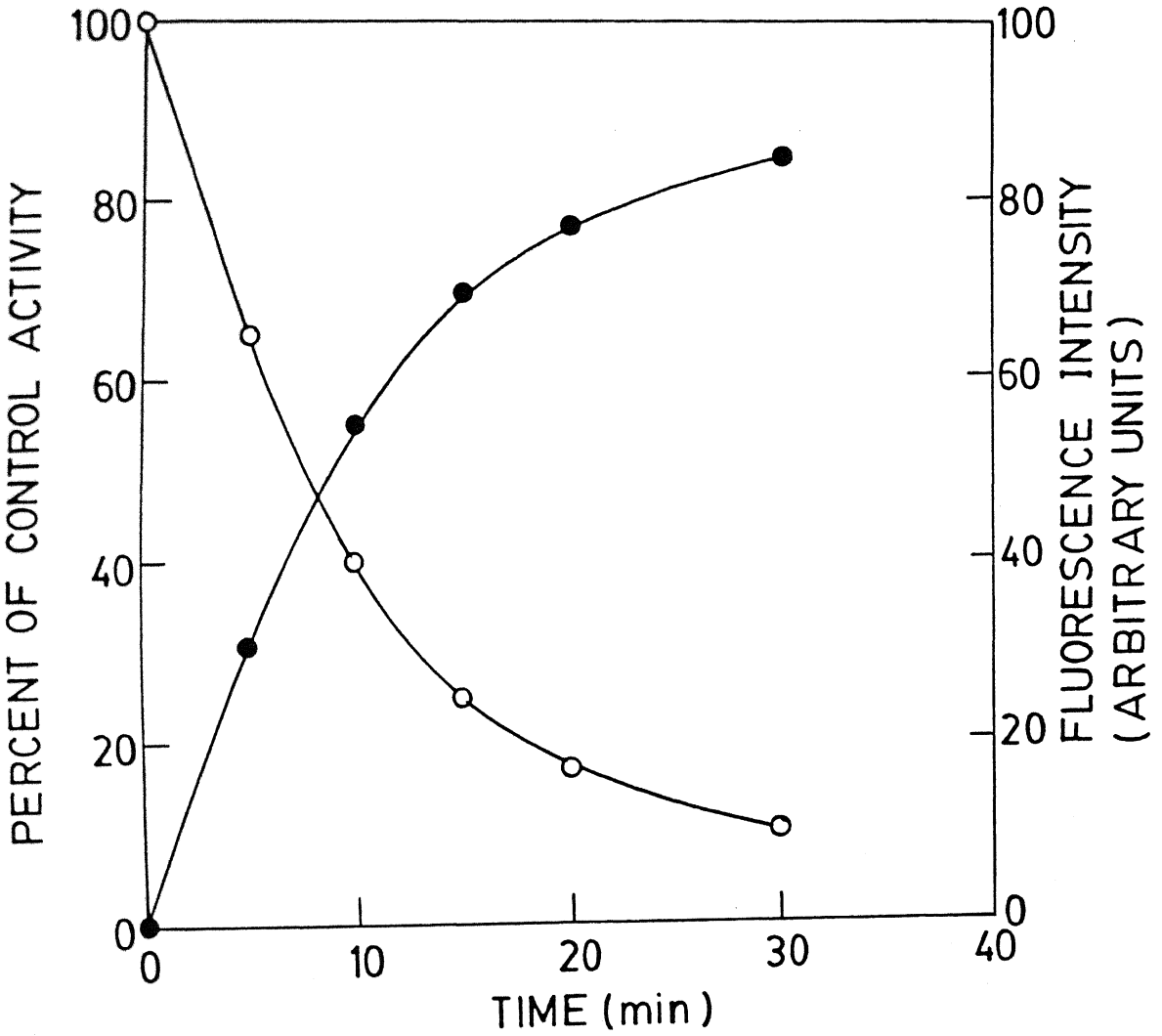


Figure V.2. Time course of inactivation of dextransucrase (O) and increase in fluorescence intensity (●) on excitation at 337 nm on treatment with *o*-phthalaldehyde. Dextransucrase (1.2 mg/ml) was incubated with 5 mM *o*-phthalaldehyde in 0.2 M acetate buffer (pH 5.2). In another set enzyme activity was measured as described in Materials and Methods.

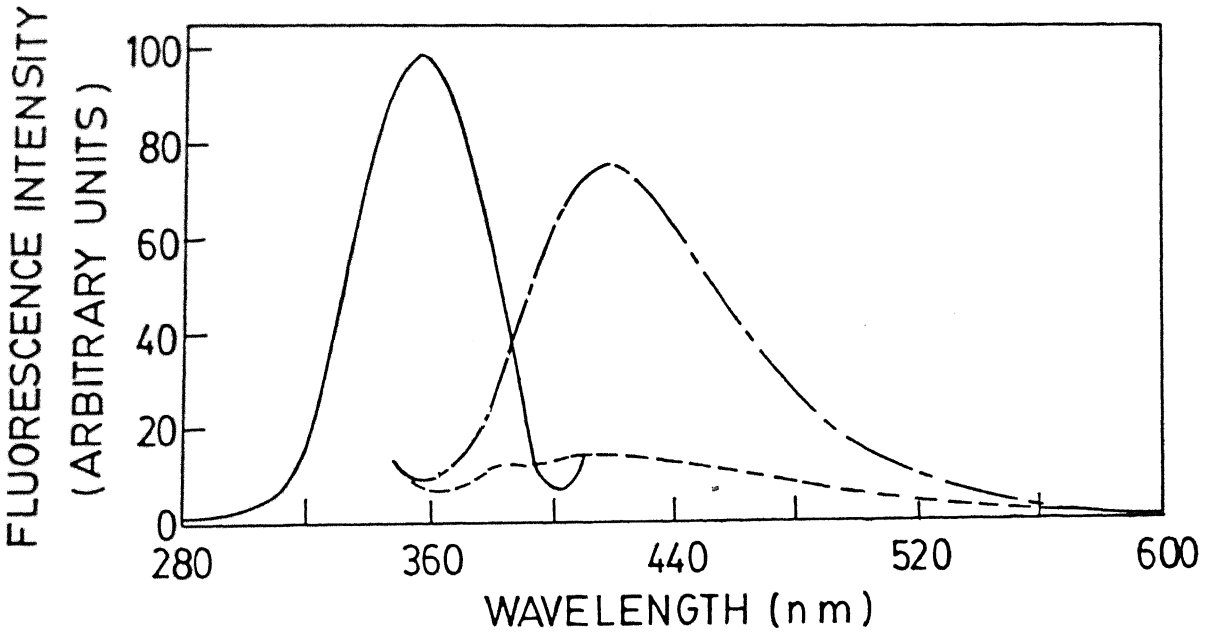


Figure V.3. Fluorescence emission spectrum on excitation at 337 nm (—- —) and fluorescence excitation spectrum (λ_{em} = 417 nm) (———) of the dextransucrase-*o*-phthalaldehyde adduct and the emission spectrum of pure enzyme (-----) on excitation at 337 nm. Dextransucrase (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 5 mM *o*-phthalaldehyde at 30°C for 30 min. For details, see "Materials and Methods".

V.3.3 Spectral analysis of modified dextransucrase by o-phthalaldehyde in presence and absence of β-mercaptoethanol

A fluorescence emission spectra of *o*-phthalaldehyde modified dextransucrase showed a maxima at 417 nm (λ_{em}) upon excitation at 337 nm (Fig. V.4). This was consistent with the formation of isoindole derivative which involves the participation of proximal thiol and ε-amino groups of cysteine and lysine, respectively [14,15]. The molar transition energy (E_T) was calculated by the following equation [14].

$$E_T = 2.985\lambda_{em} - 1087.28$$

E_T for this system was found to be 157.5 kJ/mol. The value of the molar transition energy of dextransucrase and *o*-phthalaldehyde adduct is close to that of synthetic isoindole in dioxane [14,15] indicating that the micro environment around the cysteine and lysine residues involved in isoindole formation is relatively hydrophobic in nature. The fluorescence emission spectra of the enzyme modified with *o*-phthalaldehyde in presence of β-mercaptoethanol showed a maxima at 446 nm (λ_{em}) upon excitation at 337 nm (Fig. V.4). The value of E_T for this system 244 kJ/mol was closer to the value of isoindole in methanol [14,15] showing that the isoindole derivatives resulting from the modification of enzyme in the presence of β-mercaptoethanol are in hydrophilic environment.

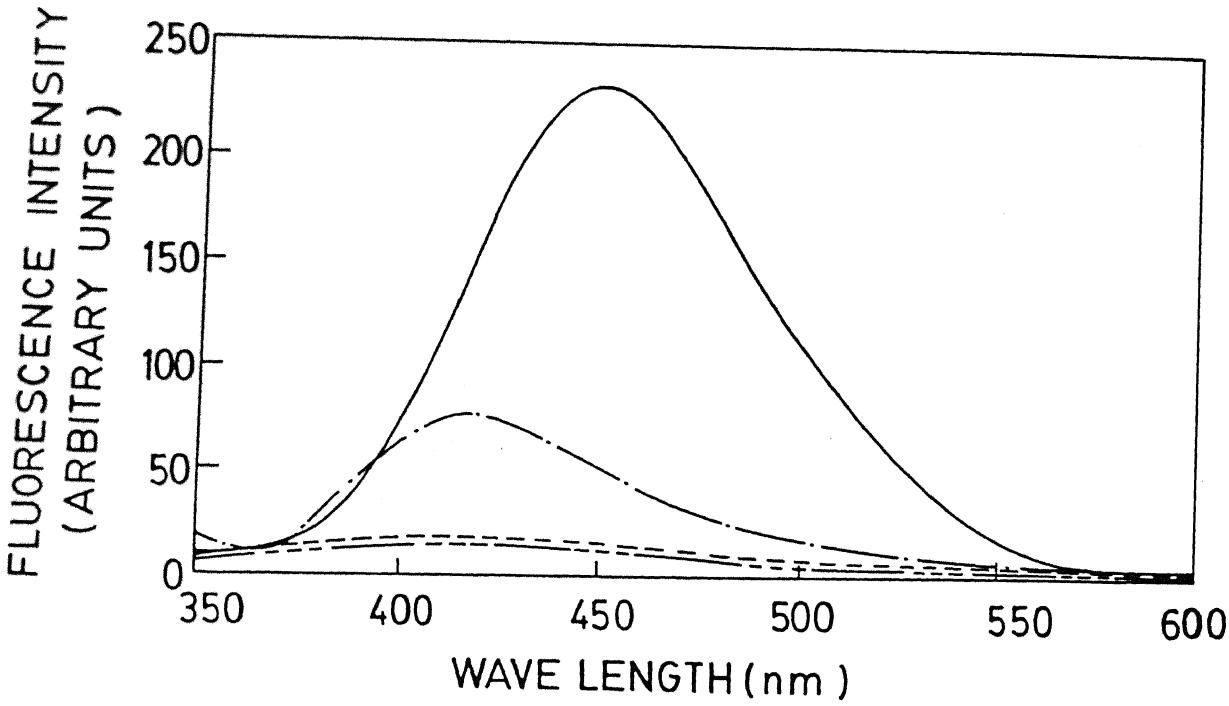


Figure V.4. Fluorescence emission spectra of dextransucrase-*o*-phthalaldehyde adduct in presence and absence of β-mercaptoethanol. The enzyme (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 5 mM *o*-phthalaldehyde for 30 min at 30°C. In another set the enzyme preincubated with 5 mM β-mercaptoethanol was treated with 5 mM *o*-phthalaldehyde. The resulting isoindole derivatives were characterized by fluorescence emission spectrum in presence (———) and absence (— - —) of β-mercaptoethanol with excitation wave length at 337 nm. The emission spectra of pure enzyme (- - - -) and the enzyme in presence of β-mercaptoethanol (- - - - -) were recorded with excitation at 337 nm. For details see "Materials and Methods".

Spectrofluorometric results with dextransucrase and o-phthalaldehyde indicated the presence of a relatively hydrophobic environment at the active site. The hydrophobicity at the active site of dextransucrase renders the ε-amino group of lysine to remain in deprotonated form to permit reaction with o-phthalaldehyde [21]. The pk_a determination for the reaction of dextransucrase-o-phthalaldehyde probably could have provided more information. But, pH dependence of inactivation process could not be determined as the enzyme itself to a large extent lost the activity, even at slightly higher or a lower pH than 5.2. However, the formation and characterization of isoindole derivative at pH 5.2 suggested that ε-amino group of lysine participating in o-phthalaldehyde reaction is in deprotonated form, corroborating the hydrophobic nature of the micro environment of lysine at the active site.

V.3.4 Kinetics of dextransucrase inactivation by o-phthalaldehyde in presence and absence of β-mercaptoethanol

It has been reported earlier that ε-amino group of lysine reacts with o-phthalaldehyde in the presence of β-mercaptoethanol and forms isoindole derivative [34]. When the modification of dextransucrase with o-phthalaldehyde was carried out in the presence of β-mercaptoethanol a significant increase in the absorbance at 337 nm was observed as compared to the modification in absence of β-mercaptoethanol (Fig. V.5).

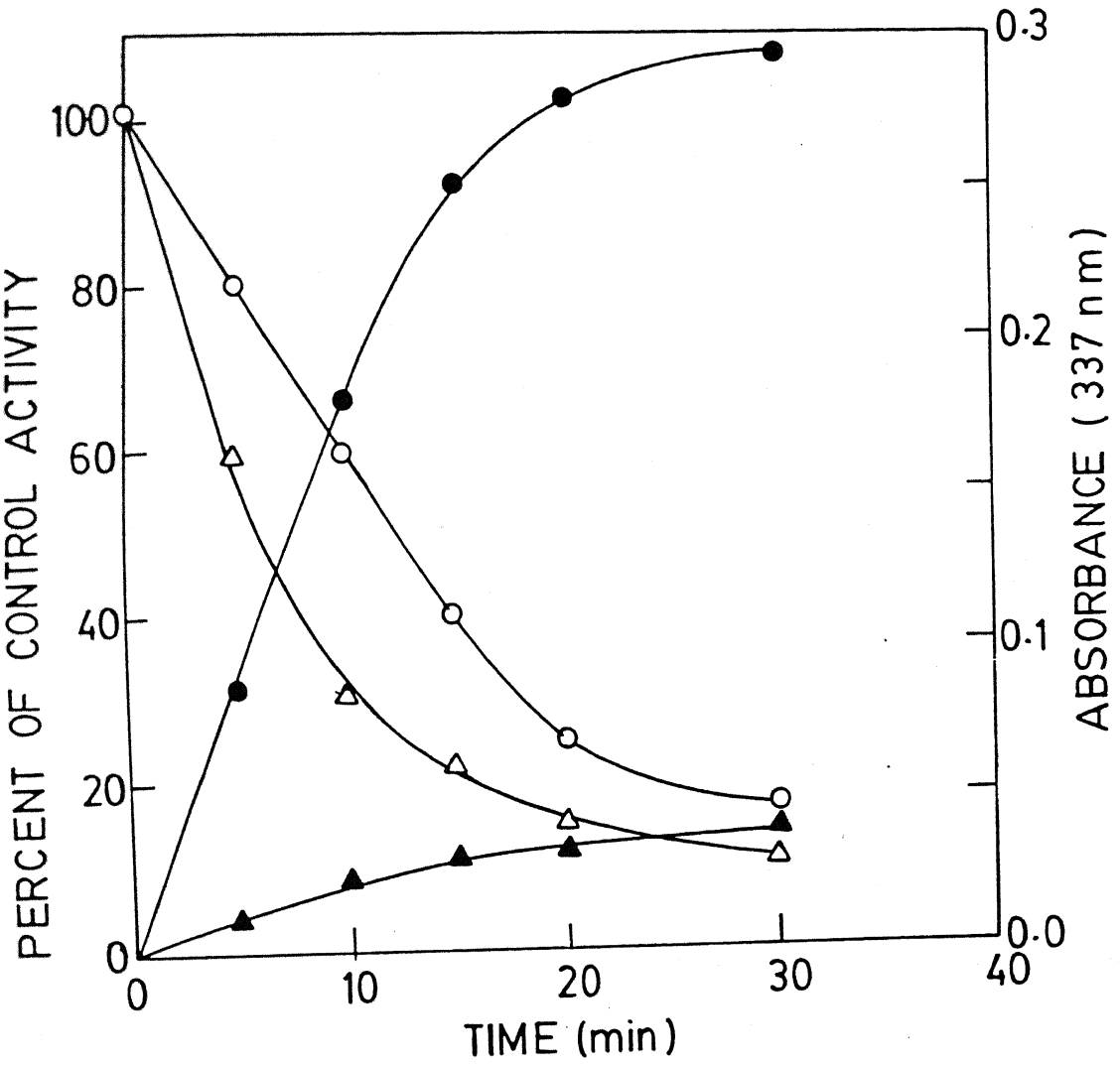


Figure V.5. Correlation of *o*-phthalaldehyde inactivation of dextransucrase with the increase in absorbance at 337 nm in absence and presence of β-mercaptoethanol. The enzyme (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) reacted with 5 mM *o*-phthalaldehyde at 30°C. The reaction was monitored continuously by recording absorbance at 337 nm in absence (▲) and presence (●) of 5 mM β-mercaptoethanol. In a separate set the reaction was monitored by assaying the enzyme activity in absence (Δ) and presence (○) of 5 mM β-mercaptoethanol as described in "Materials and Methods".

This indicated that many more lysine residues are modified in the presence of thiol group containing compound. However, kinetics of the enzyme inactivation showed that the rate of inactivation was not significantly different in absence ($k_{obs} = 5.2X10^{-3}$ min^{-1}) and that in presence of β-mercaptoethanol ($k_{obs} = 4.6X10^{-3}$ min^{-1}). These observations indicated that the lysine residues which could be modified only in the presence of β-mercaptoethanol did not contribute to loss of enzyme activity. It is the only lysine residue that is present in close proximity of the cysteine and involved in the isoindole derivative formation is critical for the activity of the enzyme.

V.3.5 Effect of substrates on dextransucrase inactivation by o-phthalaldehyde

The conventional protection experiments with only sucrose were not possible, as dextransucrase undergoes single substrate reaction. However, the property of enzyme inhibition by EDTA and reversal by Ca^{2+} ions was utilized for determining the effect of sucrose on o-phthalaldehyde inactivation [1]. The enzyme on incubation with 50 mM EDTA at 30°C resulted in 55% loss of activity in 30 min (Table V.1). The addition of 50 mM Ca^{2+} to the EDTA-inactivated dextransucrase resulted in almost complete reactivation of the enzyme. This approach was used to carry out the protection experiments, in presence of sucrose by

quenching the activity of the enzyme and then reactivating it. The results of the effect of sucrose on dextransucrase inactivation by *o*-phthalaldehyde are shown in Table V.1. A concentration of 300 mM of sucrose provided the maximum protection to the enzyme against inactivation by *o*-phthalaldehyde. Protection of enzyme by sucrose from inactivation was further corroborated by the decrease in the fluorescence intensity (Fig. V.6).

Table V.1

Effect of sucrose and glucose on dextransucrase modification by *o*-phthalaldehyde. The reagents shown in the table were incubated with the enzyme (1.2 mg/ml) for indicated time period followed by incubation with 5 mM *o*-phthalaldehyde for 30 min. Appropriate controls in each case without *o*-phthalaldehyde were run concurrently. For details see Section V.2.5.

Reagent	Control activity (%)
None	10
EDTA (50 mM, 30 min)	45
Sucrose (100 mM, 15 min)	90
Sucrose (200 mM, 15 min)	93
Sucrose (300 mM, 15 min)	97
Glucose (50 mM, 15 min)	85
Glucose (100 mM, 15 min)	95

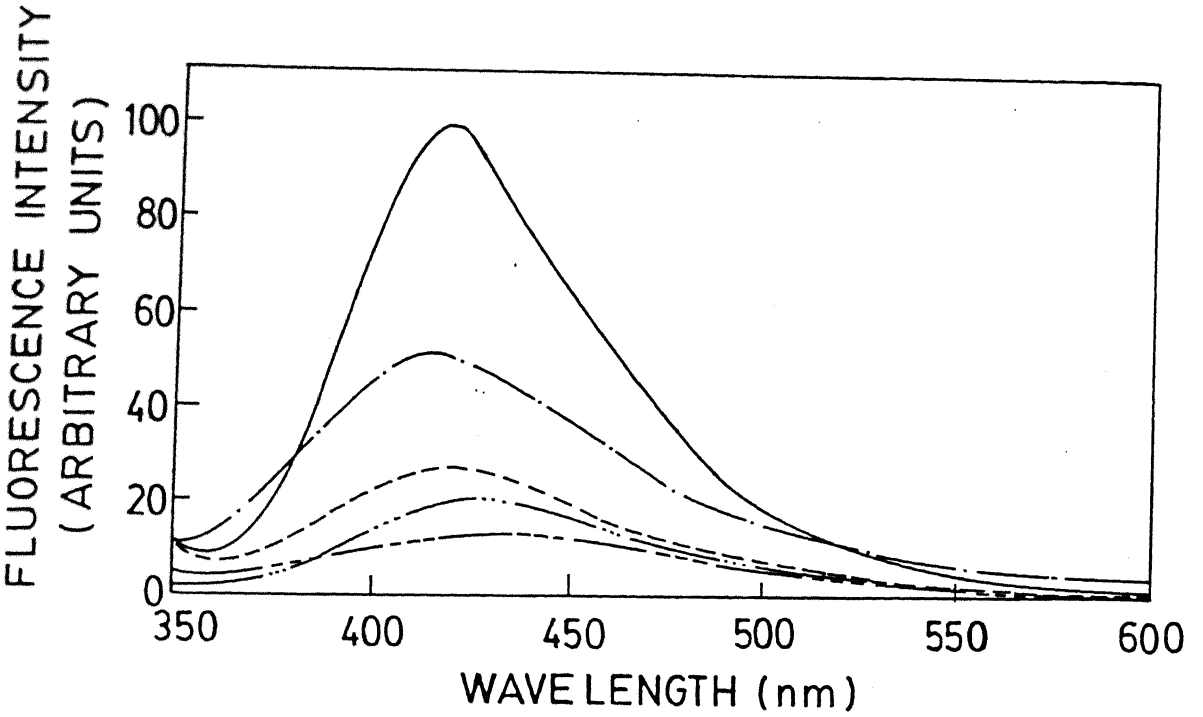


Figure V.6. Effect of substrate sucrose and denaturants on the binding of o-phthalaldehyde with dextransucrase. The enzyme (1.2 mg protein/ml) was pre-incubated with 50 mM EDTA for 30 min, followed by incubation with 300 mM substrate sucrose for 15 min and finally with 5 mM *o*-phthalaldehyde in 0.2 M acetate buffer (pH 5.2) for 30 min at 30°C. A control was run in parallel without the sucrose. The fluorescence spectra of the control (———) and with 300 mM sucrose (—-——) were recorded at excitation wave length 337 nm. The emission spectra of pure enzyme (—-—-—) and the enzyme denatured by urea (-----) or heat (—•••—) followed by *o*-phthalaldehyde treatment are also shown in the figure. For details see "Materials and Methods".

D-glucose has been reported to be an acceptor substrate and also used as the active site protecting reagent for dextransucrase [35]. D-glucose is non-reactive (in the absence of sucrose) and meets the structural requirements for binding to the active site of the enzyme. Approximately, 95% of the enzyme activity was retained in the presence of 100 mM glucose against *o*-phthalaldehyde inactivation (Table V.1). These results indicated that the inactivation of dextransucrase by *o*-phthalaldehyde has resulted from the modification of lysine and cysteine residues located at the active site of the enzyme.

V.3.6 Stoichiometry of the reaction of dextransucrase with o-phthalaldehyde

The stoichiometry of inhibition by *o*-phthalaldehyde was determined by absorbance increase at 337 nm. The percentage of residual dextransucrase activity was plotted as a function of amount of *o*-phthalaldehyde incorporated per mol of the enzyme (Fig. V.7). Approximately, 1 mol of isoindole derivative was formed per mol of enzyme when the data were extrapolated to zero enzyme activity.

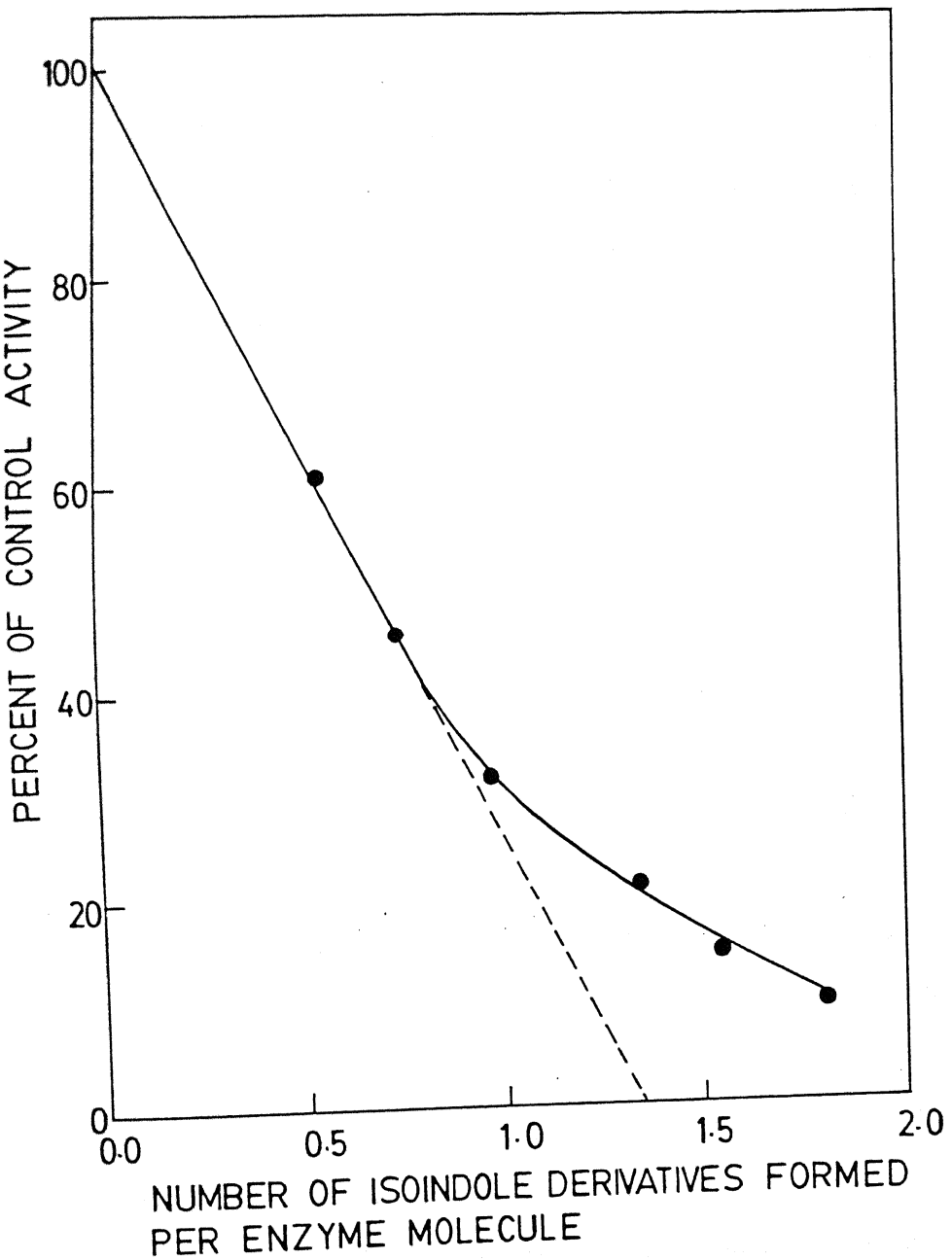


Figure V.7. Stoichiometry of inactivation of dextransucrase by *o*-phthalaldehyde. The enzyme (1.2 mg protein/ml) was incubated with 5 mM *o*-phthalaldehyde in 0.2 M acetate buffer (pH 5.2). The number of isoindole derivative formed at different time intervals during enzyme modification process was determined by taking the absorbance at 337 nm. In a parallel experiment the aliquots were withdrawn from the incubation mixture at various times and the residual activity was determined as described in "Materials and Methods".

V.3.7 Quantitative analysis of the reaction of dextransucrase with o-phthalaldehyde in presence of β-mercaptoethanol

The stoichiometry of inhibition by *o*-phthalaldehyde was determined by absorbance increase at 337 nm. It was found that 90% of inactivation occurred in 30 min of incubation in presence or absence of β-mercaptoethanol. The number of mol of isoindole derivatives formed per mol of enzyme in absence of β-mercaptoethanol was 1.8, whereas, in the presence of β-mercaptoethanol 14 mol of isoindole derivatives per mol of dextransucrase were formed (Table V.2). An increase in the enzyme concentration also increased the absorbance linearly, which further confirmed the above stoichiometry.

The stoichiometry of the reactions of dextransucrase with pyridoxal 5′-phosphate and 2,4,6-trinitrobenzenesulfonic acid have shown the presence of one critical lysine residue per enzyme molecule (Chapter IV). The amino acid composition of dextransucrase showed that there are only 1.5(±0.3) cysteine residues per molecule of enzyme (Table III.5). The results from Ellman's method [31] also showed the existence of 1.2 sulfhydryl groups per enzyme molecule. The reaction of dextransucrase with *o*-phthalaldehyde in the presence of β-mercaptoethanol leads to the modification of 14 lysine residues as shown by stoichiometric results (Table V.2). However, the rates of enzyme inactivation were not significantly different in the absence or presence of

β-mercaptoethanol (Fig. V.5). This clearly demonstrated that lysine residues which are modified in the presence of β-mercaptoethanol do not play a role in enzyme inactivation. Thus only one specific lysine residue which is proximal to cysteine is essential for the activity of dextransucrase.

Table V.2

Quantitative analysis of dextransucrase inactivation by *o*-phthalaldehyde in absence and presence of β-mercaptoethanol (BME). Different concentrations of dextransucrase were incubated with 5 mM *o*-phthalaldehyde for 30 min, which resulted in 90% of inactivation of the enzyme. The number of isoindole derivative formed per dextransucrase molecule was calculated as described in Section V.2.6.

Concentration of dextransucrase	Absorbance at 337 nm		Number of isoindole derivatives formed/enzyme molecule	
(μM)	No BME	BME	No BME	BME
1.0	0.013	0.105	1.7	13.7
2.7	0.038	0.295	1.8	14.1
4.0	0.055	0.441	1.8	14.3
4.8	0.067	0.512	1.8	13.9

V.3.8 Characterization of the cysteine and lysine residues involved in o-phthalaldehyde reaction

The enzyme showed no inactivation on treatment with thiol specific inhibitors. The enzyme on incubation with DTNB for 15 min followed by the incubation with *o*-phthalaldehyde for 30 min lost 85% of its activity (Table V.3). This clearly showed that even in the presence of DTNB, *o*-phthalaldehyde was able to bind to the lysine leading to the inactivation of the enzyme. It was interesting to note that dextransucrase preincubated with DTNB followed by treatment with *o*-phthalaldehyde did not show any fluorescence on excitation at 337 nm. This was due to the binding of DTNB to cysteine thereby preventing the formation of isoindole derivative. These results indicated that the cysteine is present in close proximity of the lysine and is involved in the isoindole derivative formation but is not critical for the activity of enzyme.

It has been reported that *o*-phthalaldehyde can react with primary amino group in the absence of thiol compounds leading to the formation of non-fluorescent products [34]. The results suggested that *o*-phthalaldehyde reacted with the specific lysine residue, when the cysteine was blocked by DTNB to give non-fluorescent adduct, which inactivated the enzyme. However, in the absence of DTNB, inactivation of dextransucrase by o-phthalaldehyde showed a good correlation with isoindole formation (Fig. V.2). These results clearly indicated that the

cysteine is present in close proximity of the lysine and is not essential for the activity of the enzyme.

Table V.3

Effect of DTNB preincubation of dextransucrase on inactivation by o-phthalaldehyde. The enzyme (1.2 mg/ml) preincubated with DTNB in 0.2 M acetate buffer (pH 5.2) at 30°C was followed by the treatment with *o*-phthalaldehyde for indicated time interval. Aliquots (0.2 ml) from incubation mixtures were analyzed for the enzyme activity as described in Section V.2.7.

Reagent	Control Activity (%)
None	100
o-phthalaldehyde (5mM, 30min)	10
DTNB (5mM, 30min)	95
DTNB (5mM, 15min) + *o*-phthalaldehyde (5mM, 30min)	15

Experiments with prior incubation of dextransucrase with PLP followed by *o*-phthalaldehyde incubation, were performed in order to ascertain whether these two inhibitors are binding to same lysine residue that leads to enzyme inactivation. It is generally known that the inhibition by PLP can be reversed by dilution or dialysis. The enzyme on incubation with PLP followed by *o*-phthalaldehyde without terminating the reaction by cysteine and β-mercaptoethanol, did not show any

fluorescence but after dialysis it showed the fluorescence emission maxima at 417 nm with much reduced intensity as compared to the control (Fig V.8). Same results were obtained even when lower concentrations of PLP were used. This observation indicated that both the inhibitors are binding to the same lysine residue that is involved in catalytic activity of dextransucrase. All these results led to the conclusion that there is one lysine residue which is essential for the enzyme for its activity.

V.3.9 Effect of enzyme denaturation on the modification by o-phthalaldehyde

Dextransucrase when treated with urea or heat followed by incubation with *o*-phthalaldehyde, showed a marked decrease in the fluorescence emission intensity as compared to the native enzyme *o*-phthalaldehyde adduct (Fig. V.6). Thus, these results clearly indicated that proximal integrity of the lysine and cysteine residues at the active site of the native enzyme is essential for *o*-phthalaldehyde reaction.

The isoindole derivative formation need not depend on the closeness of lysine and cysteine residues in the primary structure, but, rather on the specific proximity of these residues in the tertiary structure of the enzyme [18]. The affinity of *o*-phthalaldehyde binding with denatured dextransucrase was lowered as compared to the native form of

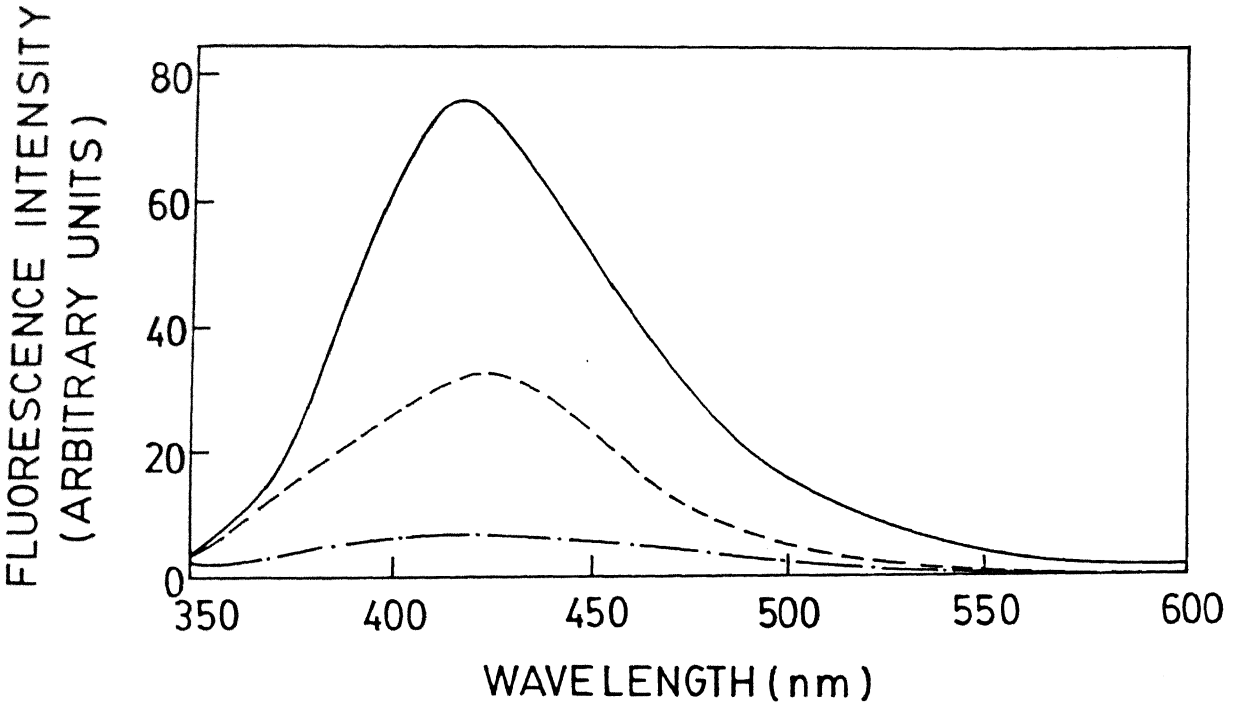


Figure V.8. Effect of PLP pretreatment of dextransucrase on fluorescence emission spectra of dextransucrase-*o*-phthalaldehyde adduct. The enzyme (1.2 mg protein/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 30 mM PLP for 1h followed by the treatment with 5 mM *o*-phthalaldehyde for 30 min at 30°C. The fluorescence emission spectra were recorded at excitation wave length 337 nm before (—·—) and after (-----) the dialysis of incubation mixture. A control (———) with the enzyme treated with 5 mM *o*-phthalaldehyde for 30 min was run in parallel without PLP pretreatment.

enzyme. Thus, it can be concluded that alteration in the native conformation of the enzyme disrupts the favorable orientation of lysine and cysteine residues at the catalytic center for the *o*-phthalaldehyde reaction.

V.4 SUMMARY

Chemical modification reagents can be used successfully to identify amino acid residues that are important for enzyme activity provided that the following two criteria are fulfilled: First, chemical modification of these amino acid residues must result in a loss of activity. Second, this inactivation must be prevented by substrates or substrate analogs. Dextransucrase was rapidly inactivated by *o*-phthalaldehyde and the inactivation followed pseudo-first order kinetics. Spectrofluorometric results with dextransucrase and *o*-phthalaldehyde indicated the presence of a relatively hydrophobic environment at the active site. The substrate sucrose and acceptor substrate glucose protected the enzyme against *o*-phthalaldehyde inactivation indicating that the lysine and cysteine residues are present at the active site. The stoichiometry of dextransucrase-*o*-phthalaldehyde reaction showed that one isoindole derivative is formed per enzyme molecule. The reaction of *o*-phthalaldehyde with enzyme in the presence of β-mercaptoethanol leads to the modification of 14 lysine residues. However, the rate of inactivation was

marginally higher in the absence of β-mercaptoethanol. This clearly demonstrated that lysine residues which are modified in the presence of β-mercaptoethanol do not play any role in the enzyme inactivation. Thus only one specific lysine residue which is proximal to cysteine residue, is essential for the activity of dextransucrase.

Dextransucrase was not inactivated by thiol specific inhibitors indicating that, cysteine involved in *o*-phthalaldehyde reaction leading to isoindole formation is not required for the activity. Further, the dextransucrase on preincubation with DTNB followed by treatment with *o*-phthalaldehyde did not exhibit any fluorescence on excitation at 337 nm. However, the inactivation of the enzyme was still observed as *o*-phthalaldehyde was able to react with the specific lysine residue and form non-fluorescent product, when the proximal cysteine was blocked by DTNB. Dextransucrase pretreated with PLP followed by *o*-phthalaldehyde incubation showed that both these inhibitors are binding to the same specific lysine residue that is essential for the enzyme activity. All these results led to the conclusion that there is one lysine residue which is essential for the enzyme for its activity. Denaturation of dextransucrase by urea or heat treatment prior to *o*-phthalaldehyde addition resulted in the decrease of fluorescence intensity indicating that the native conformation of the enzyme is essential for isoindole

REFERENCES

1. Robyt, J.F. and Walseth, T.F. (1979) Carbohydr. Res. 68, 95-111.
2. Kobayashi, M. and Matsuda, K. (1980) Biochim. Biophys. Acta 614, 46-62.
3. Monsan, P., Paul, F., Lopez, A. (1987) Methods Enzymol. 136, 239-254.
4. Fu, D. and Robyt, J.F. (1990) Prep. Biochem. 20, 93-106.
5. Goyal, A. and Katiyar, S.S. (1994) J. Microbiol. Methods 20, 225-231.
6. Robyt, J.F., Kimble, B.K. and Walseth, T.F. (1974) Arch. Biochem. Biophys., 165, 634-640.
7. Kobayashi, M., Yokoyama, I. and Matsuda, K. (1986) Agric. Biol Chem. 50, 2585-2590.
8. Tanriseven, A. and Robyt, J.F. (1993) Carbohydr. Res. 245, 97-104.
9. Abo, H., Matsumura, T, Kamada, T., Ohta, H., Fukui, K., Kato, K. and Kagawa, H. (1991) J. Bacteriol. 173, 989-996.
10. Su, D. and Robyt, J.F. (1994) Arch. Biochem. Biosphys., 308, 471-476.
11. Mooser, G., Hefta, S.A., Paxton, R.J., Shively, J.E. and Lee, T.D. (1991) J. Biol. Chem., 266, 8916-8922.
12. Fu, D. and Robyt, J.F. (1988) Carbohydr. Res., 183, 97-109.
13. Blaner, W. S. and Churchich, J. (1979) J. Biol. Chem. 254, 1794-1798.

14. Palczewski, K., Hargrave P.A. and Kochman, M. (1983) Eur. J. Biochem. 137, 429-435.

15. Puri, R.N., Bhatnagar, D. and Roskoski, R., Jr. (1985) Biochemitsry, 24, 6499-6508.

16. Puri, R.N., Bhatnagar, D. and Roskoski, R., Jr. (1985) Biochemitsry, 24, 6508-6514.

17. Puri, R.N., Bhatnagar, D. and Roskoski, R., Jr. (1988) Biochim. Biophys. Acta, 957, 34-46.

18. Bhagwat, A.S. and Gopala Krishna, K. (1986) Biochim. Biophys. Acta, 873, 45-52.

19. Rider, M.H. and Hue, L. (1989) Biochem. J. 262, 97-102.

20. Maralihalli, G. B. and Bhagwat, A. S. (1990) Ind. J. Biochem. Biophys. 27, 141-145.

21. Huynh, Q.K. (1990) J. Biol. Chem. (1990) 265, 6700-6704.

22. Chen, C.-Y., Emig, F.A., Schramm, V.L. and Ash, D.E. (1991) J. Biol. Chem. 266, 16645-16652.

23. Sheikh, S. and Katiyar, S.S. (1992) Biochem. Int. 27, 517-524.

24. Sheikh, S. and Katiyar, S.S. (1993) Biochim. Biophys. Acta, 1203, 276-281.

25. Sheikh, S. and Katiyar, S.S. (1993) Biochem. Molec. Biol. Int., 29, 719-727.

26. Sheikh, S. and Katiyar, S.S. (1994) J. Enz. Inhibition 8, 39-50.

27. Goyal, A. and Katiyar, S.S. (1995) Biochem. Molec. Biol. Int. (in press).

28. Nelson, N., (1944) J. Biol. Chem., 153, 375-380.

29. Somogyi, M. (1945) J. Biol. Chem., 160, 69-73.

30. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

31. Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.

32. Salnikow, J., Liao, T.-H., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 1480-1488.

33. Goodwin, W.T. and Morton, R.A. (1946) Biochem. J. 628-632.

34. Simons, S.S. (Jr.) and Johnson, D.F. (1978) J. Org. Chem. 43, 2886-2891.

35. Kobs S.F. and Mayer R.M. (1991) carbohydr. Res. 211, 317-326.

CONCLUSIONS

The studies presented in the thesis are primarily focused on the maintenance of *Leuconostoc mesenteroides* NRRL B-512F, optimization of conditions for production of dextransucrase, effect of some nutrients on enzyme production, purification methods based on fractionation and phase-partition using polyethylene glycols, effect of certain stabilizers of enzyme and active site mapping of dextransucrase using chemical modification studies. The results of studies carried out have led us to draw following important conclusions:

1. Modified *Lactobacillus* MRS was found to be the most suitable medium for maintenance and propagation of *Leuconostoc mesenteroides* NRRL B-512F which gave higher enzyme yield as compared to other maintenance media, when the culture was transferred to enzyme production medium.
2. The optimum temperature of 23°C and still flask culture were found to be the most suitable conditions for production of dextransucrase.
3. Dextransucrase showed maximum activity at a temperature, 30°C, pH, 5.2 and sucrose concentration, 10%.

4. The addition of certain nutrients to the enzyme production medium such as beef extract, peptone, tween 80, $MgCl_2$ and NaF enhanced the dextransucrase production.
5. A three consecutive steps fractionation, using a final concentration of 33% PEG 400 reproducibly yielded a homogeneous preparation of dextransucrase, showing a single band on analysis by SDS-polyacrylamide gel electrophoresis. The purification method gave enzyme protein having a specific activity of 29 U/mg protein and an overall yield over 70%.
6. In a three successive steps of phase-partition by PEG 6000 a specific activity of 38.7 U/mg was obtained after the third step, with an overall yield of 78%.
7. The molecular weight of the purified dextransucrase on analysis by SDS-polyacrylamide gel electrophoresis was found to be 188,00.
8. The amino acid composition of purified dextransucrase was detremined. The enzyme was rich in glycine and contained only 1.5±0.3 cysteine residues per enzyme molecule.
9. Glycerol proved to be the best stabilizer of enzyme against activity losses among glycerol, PEG 8,000, dextran and tween 80.
10. Fluorescence spectra of PLP modified enzyme showed that the inactivation is due to binding of PLP with ε-amino group of lysine residue.

11. Fluorescence emission spectra of *o*-phthalaldehyde modified dextransucrase showed the formation of an isoindole derivative that involves proximal thiol and ε-amino groups of cysteine and lysine, respectively.
12. Sucrose and glucose protected the enzyme against PLP and *o*-phthalaldehyde inactivation, indicating that the inactivation has resulted from the modification of a lysine residue present at the active site of enzyme.
13. The stoichimetric results with all the three inhibitors showed that one lysine residue present at the active site which is essential for the activity dextransucrase.
14. Dextransucrase was not inactivated by DTNB, showing that cysteine is present in close proximity of the lysine is not essential for the enzyme activity.
15. Fluorescence studies using PLP and *o*-phthalaldehyde showed that both the inhibitors bind to the same lysine.
16. Chemical modification studies on dextransucrase using PLP and TNBS acid, convincingly demonstrated the presence of an essential lysine residue at the active site.
17. The results of dextransucrase inhibition studies by *o*-phthalaldehyde showed that the proximal cysteine is not essential for activity of enzyme, whereas, the lysine residue is essential for the activity of enzyme.

LIST OF RESEARCH PUBLICATIONS

1. Fractionation of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase by polyethylene glycol: A simple and effective method of purification.

   Arun Goyal and S.S. Katiyar (1994) Journal of Microbiological Methods 20, 225-231.

2. Inactivation of dextransucrase of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase by specific modification of lysine residues with pyridoxal 5′-phosphate.

   Arun Goyal and S.S. Katiyar (1995) Journal of Enzyme Inhibition, 8(4), 291-295.

3. Involvement of a lysine residue in the inactivation of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase by o-phthalaldehyde.

   Arun Goyal and S.S. Katiyar (1995) Biochemistry & Molecular Biology International, (in press)

4. Optimal conditions for production of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase and its properties.

   Arun Goyal, Manisha Nigam and S.S. Katiyar (1995) Journal of Basic Microbiology, (accepted)

5. 2,4,6-Trinitrobenzenesulphonic acid as a probe for lysine at the active site of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F.

   Arun Goyal and S.S. Katiyar (1995) Biochemistry & Molecular Biology International, (communicated)

6. Dependence of dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F on maintenance media.

   Arun Goyal and S.S. Katiyar (1995) Journal of Basic Microbiology, (communicated)

7. Chemical modification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by pyridoxal 5′-phosphate and 2,4,6-trinitrobenzenesulphonic acid: Evidence for the presence of an essential lysine at the active site.

   Arun Goyal and S.S. Katiyar (1995) Archives of Biochemistry & Biophysics, (communicated)

8. Studies on the inactivation of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase by o-phthalaldehyde: Evidence for presence of an essential lysine at active site.

   Arun Goyal and S.S. Katiyar (1995) European Journal of Biochemistry, (communicated)

VITAE

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